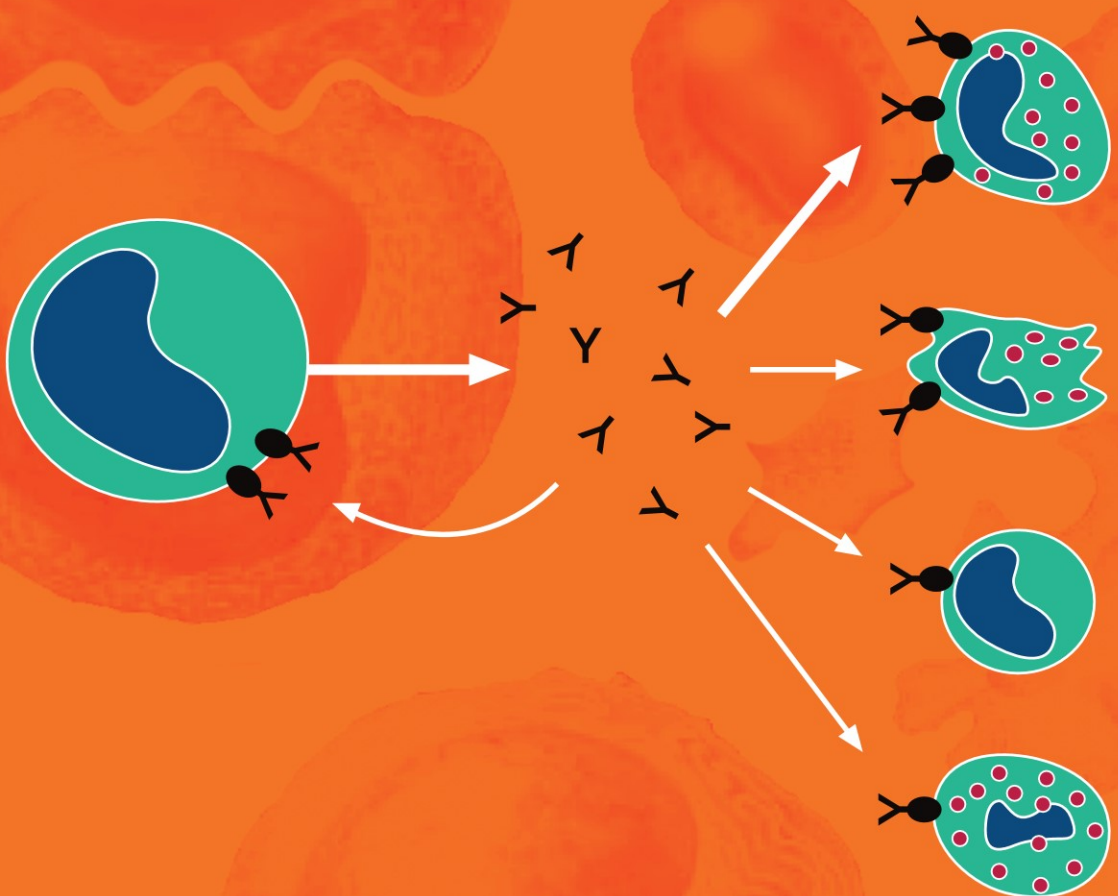
Principles of Immunopharmacology

3rd revised and extended edition



Edited by
Frans P. Nijkamp and
Michael J. Parnham

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Preface to the first edition

The rapid developments in immunology in recent years have dramatically expanded our knowledge of mammalian host defence mechanisms. The molecular mechanisms of cellular interactions during immune responses have been unravelled, the intracellular responses involved in signal transduction delineated and an ever-increasing number of soluble mediators of immune and inflammatory reponses have been discovered.

The initial result of this explosion of knowledge has been to provide the researcher and the clinician with an arsenal of diagnostic tools with which the immunological bases of disease processes can be investigated. This has made disease diagnosis much more precise, enabling the physician to tailor therapy much more closely to the individual patient's needs. However, better understanding of disease processes only provides a gradual improvement in therapy. This is because the new molecular targets that have been uncovered must first be tested as potential bases for immunomodulatory drug actions and then the new compounds must be subject to extensive development studies. As a result of the molecular unravelling of the immune system, we now understand more precisely the mechanisms of action of some established therapies, such as anti-allergic and anti-asthma agents, including the corticosteroids. New rational treatments based on molecular mechanisms also are now entering clinical practice and are making their mark on cancer, infectious and autoimmune disease therapy.

Concomitantly with these advances in understanding of molecular mechanisms of immunity, immunodiagnosis and immunotherapy, it has become possible to test more accurately the way in which a variety of drug classes interact with the immune system. It is of particular importance to regulatory authorities that the toxic side-effects of immunomodulatory drugs can be distinguished from their beneficial therapeutic effects.

Currently, it is only possible to obtain an overview of these various aspects of immunopharmacology by reading a range of immunological, pharmacological, diagnostic and toxicological literature. Good immunological textbooks are available, while immunopharmacology is covered mainly in terms of the inflammatory response. *Principles of Immunopharmacology* is intended to provide for the first time in a single volume a basic understanding of immunological mechanisms, a review of important immunodiagnostic tools and a description of the main pharmacological agents which modify the immune response, together with an introduction to immunotoxicology. As such we hope that it will be useful as a reference text for physicians, researchers and students with a rudimentary knowledge of immunology.

We, the editors, are grateful to all the authors who have invested their time and effort into this volume. We have received continuous help and encouragement from Petra Gerlach and Katrin Serries of Birkhäuser Verlag and particular thanks are due to Dinij van der Pal for administrative assistance.

Frans P. Nijkamp
Michael J. Parnham
March, 1999

Preface to the second edition

Our knowledge of immunological processes and their modulation has progressed considerably since the first edition of *Principles of Immunopharmacology*. Molecular mechanisms have been elucidated so that we are now in a position to understand many of the complex pathways leading from surface stimulation to cellular responses. We now appreciate much better that the innate immune response is also regulated by far more external and internal stimuli than was previously realised and are starting to understand the role of memory and regulatory cells. Advances in genomics and proteomics have enabled the identification of many genes and new proteins that are intimately involved in the responses of the immune system. We have sought to include the most important of these advances in the first part of this second edition of *Principles of Immunopharmacology*. In addition to including new mechanisms in the section on the immune response, we have also included the new techniques of the genomic and proteomic revolution in the diagnostics section, as methods such as microarrays have now become an essential aspect of cellular analyses.

Inevitably, our increased understanding of immune mechanisms has opened opportunities for the development of novel drugs to treat inflammation and disorders of the immune system. Biologicals are now commanding worldwide interest, both in research and development and in clinical practice. The section on therapy has now been expanded to accommodate these new therapeutic approaches, as well as describing our improved understanding of the mechanisms of action of established agents. The final section on immunotoxicology has also been updated, particularly with regard to new regulatory changes.

While maintaining the unique approach of providing sections on immunology, immunodiagnostics, therapy and immunotoxicology in a single volume, we have also introduced a new double-column format to provide easier access to the text. Important statements are highlighted and instead of giving annotations in the margins, key terms are now indicated in the text and presented in a glossary* at the end of the book. A new appendix summarises important characteristics of commercially available therapeutic agents.

We are very grateful to many of the contributors to the first edition, who have kindly revised and modified their chapters, as well as to the additional authors who have added totally new information. The preparation of this second edition has been the result of close collaboration with Dr. Hans-Detlef Klueber and his colleagues at Birkhäuser Verlag. Thanks for all your help, advice and hard work. We hope you, the reader, will find the new edition useful and informative.

Frans P. Nijkamp
Michael J. Parnham
March, 2005

* Words included in the glossary are highlighted in the text with CAPITAL LETTERS.

Preface to the third edition

The last 5–6 years since the publication of the second edition of *Principles of Immunopharmacology* have seen several notable changes in our understanding of the immune system. Not least of these has been the expansion of the number of defined T cell subsets with the rapid appreciation of their roles in infectious and autoimmune diseases. In parallel, the spotlight has fallen increasingly on the contribution of sub-populations of dendritic cells and the recognition that these cells represent potential targets for drug therapy. These areas are given detailed attention in this third edition, together with a new chapter on pre- and probiotics and a greater emphasis on therapeutic biologicals, which are providing the major thrust in new immunomodulatory drug development. All other chapters have been updated by the authors, bringing the book in line with the latest progress in each field. The format of the textbook, with sections on immune mechanisms, diagnostics, therapeutic agents and immunotoxicology, a glossary of keywords and the collection of appendices, has been retained and illustrations have been improved. An introduction has been added, providing an overview of inflammation, the immune response and its pharmacological modification as a framework for the subsequent details given in each chapter. Most of these improvements have been made in response to comments from authors and readers, which we much appreciate.

Once again, we are very grateful to all the contributors for taking the time to revise or contribute new chapters. We are particularly grateful to Els Tange-Bijl for her organisational talent and expert co-ordination of the contacts with authors and her persistence in pressing them to deliver the manuscripts. We also thank Anke Brosius and her colleagues at Springer Basel for the professional desk editing of the final text and Dr. Hans-Detlef Klueber for sustaining the publisher's support and guidance for the project. We hope you, the reader, find the final product informative and useful.

Frans P. Nijkamp
Michael J. Parnham
October, 2010

Abbreviations

A23187	calcium ionophore
AA	arachidonic acid
AAA	abdominal aortic aneurysm
Ab	antibodies, antibody
ABA	antibacterial agents
ABC transporter	ATP-binding cassette transporter
aca	anticomplementary activity
ACE	angiotensin converting enzyme
ACTH	corticotropin, adrenocorticotrophic hormone
ADCC	antibody-dependent cellular cytotoxicity
ADP	adenosine diphosphate
AFC	antibody-forming cell assay
Ag	antigen
AGP	acid glycoprotein
AgR	antigen receptor
AHA	autoimmune hemolytic anemia
AICD	activation-induced cell death
AID	activation-induced cytidine deaminase
AIDS	acquired immune deficiency syndrome
ALG	anti-lymphocyte globulin
ALL	acute lymphocytic leukemia
ALS	amyotrophic lateral sclerosis
ALXR	affinity G protein-coupled lipoxin receptors
AML	acute myeloid leukemia
AMP	antimicrobial peptide
ANC	absolute neutrophil count
ANLL	acute non-lymphocytic leukemia
ANP	atrial natriuretic peptide
AP-1	activator protein-1
AP50	complement activity that causes 50% hemolysis *via* the alternative route
APC	antigen-presenting cell
APC	allophycocyanin
APRIL	A proliferation inducing-ligand
βARK	β-adrenergic receptor kinase
ATF-2	activating transcription factor
ATG	anti-thymocyte globulin
ATL	aspirin-triggered lipoxins
ATP	adenosine triphosphate
AVP	arginine vasopressin
AZA	azathioprine
BAFF	B cell activating factor
BAGE	B antigen
BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
BCG	Bacille Calmette-Guérin
Bcl2	B cell lymphoma-2
B-CLL	B-chronic lymphocytic leukemia
BCMA	B cell maturation antigen
BCR	B cell receptor
BCR-ABL	breakpoint cluster region-Abelson
BDCA	blood dendritic cell antigen
BDP	beclomethasone dipropionate
BFU	burst-forming unit
BLNK	B cell linker protein
BLyS	B lymphocyte stimulator
BM	bone marrow
BMT	bone marrow transplantation
B-NHL	B-non-Hodgkin's lymphoma
BPI	bactericidal permeability increasing protein
BRM	biological response modifier
BT	Buehler test
Btk	Bruton's tyrosine kinase
C	constant (gene segment)
C/E	cellular/extracellular concentration ratio
C3b	large fragment of complement factor 3
C5a	small fragment of complement factor 5
Ca^{2+}	calcium ion
Ca^{2+}_i	intracellular calcium
CAD	coronary artery disease

CAM	cell adhesion molecule
CAML	calcium modulator and cyclophilin ligand
cAMP	cyclic 3',5'-adenosine monophosphate
CCD	charge-coupled device
CCL1	CC chemokine-1
CD	cluster of differentiation
CD40L	CD40 ligand
cDNA	complementary deoxyribonucleic acid
CDR	complementarity determining region
CEA	carcinoembryonic antigen
CF	cystic fibrosis
CFA	complete Freund's adjuvant
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony-forming unit
CGD	chronic granulomatous disease
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
CH_{50}	complement activity that causes 50% hemolysis *via* the classical route
CHC	chronic hepatitis C
CHMP	E.U. Committee on Human Medicinal Products
C_HN	constant domain number N of the heavy chain (N = 1, 2 or 3 for IgA, IgD and IgG and 1, 2, 3 or 4 for IgE and IgM)
CIDP	chronic inflammatory demyelinating polyneuropathy
Cl^-	chloride ion
CL	class
CML	chronic myelogenous leukemia
CMP	cytidine monophosphate
CMT	chemically-modified tetracycline
CMV	cytomegalovirus
CNS	central nervous system
COMT	catechol-*o*-methyl transferase
Con A	concanavalin A
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
CpG	cytosine-phosphate-guanosine
CpG DNA	synthetic oligodeoxyribonucleotides containing CpG-dinucleotides
CPMP	E.U. Committee on Proprietary Medicinal Products
CR	complement receptor
CRAC	Ca^{2+} release-activated Ca^{2+} current
CRH	corticotropin-releasing hormone
CRMO	chronic recurrent multifocal osteomyelitis
CRP	C-reactive protein
CsA	ciclosporin/cyclosporin A
CSAID	cytokine suppressive anti-inflammatory drugs
CSF	colony-stimulating factor
CT	chemotaxis
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen-4
CX3CL1	fractalkine
CXC or CXCL	CXC chemokine
CXCR	CXC chemokine receptor
CY	cyclophosphamide
Cy5/Cy3	cyanine dye 5 and 3
CYP	cyclophilin or cytochrome
CYP isoforms	cytochrome P450 isoforms
cysLT	cysteinyl leukotrienes
D	diversity (gene segment)
DAF	decay-accelerating protein
DAG	diacylglycerol
DAMP	damage associated molecular pattern
DAO	diamine oxidase
DC	dendritic cell
DC-SIGN	dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin
dCTP	desoxycytosine triphosphates
2DE	two-dimensional gel electrophoresis
DFS	disease-free survival
DHODH	dihydroorotate dehydrogenase
DISC	death-inducing signaling complexes
DLI	donor leukocyte infusion
DLN	draining lymph nodes
DMARD	disease-modifying anti-rheumatic drug
DN T cells	double-negative T cells
DNA	deoxyribonucleic acid
DNAM-1	DNAX accessory molecule 1
dNTP	desoxynucleoside triphosphates
DP	PGD receptor
DPB	diffuse panbronchiolitis
DPI	dry powder inhaler
DRESS	drug rash with eosinophilia and systemic symptoms
DRG	dorsal root ganglia
DTH	delayed-type hypersensitivity
DTP	diphtheria-tetanus-polio

EAE	experimental auto-immune encephalo-myelitis
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetraacetic acid
EGCG	epigallocatechin-3-gallate
EGFR	epidermal growth factor receptor
eGPx	extracellular glutathione peroxidase
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
ELK-1	member of ETS oncogene family
EM	endomorphins
EMA	European Medicines Agency
EMEA	European Agency for the Evaluation of Medicines
ENA-78	epithelial neutrophil-activating protein-78
ENC	endotoxin neutralizing capacity
eNOS	endothelial nitric oxide synthase
EP	PGE receptor
EPAC	exchange protein activated by cAMP
Ep-CAM	epithelial cell adhesion molecule
EPO	erythropoietin
E-selectin	endothelial selectin
EST	expressed sequence tags
Fab	antigen-binding fragment of immuno-globulins
Fabc	monovalent antigen-binding fragment of immunoglobulins comprising one Fab and Fc part
FasL	Fas ligand
Fc	constant fragment of immunoglobulin
Fc receptor	receptor for the constant binding fragment of immunoglobulins
FcγRI	Fc gamma receptor I
FcεRI	receptor for the Fcε part of the IgE molecule
FcγRIIb	receptor IIb for immunoglobulin G
FcRn	neonatal receptor for the Fc γ fragment of the IgG molecule
FDA	U.S. Food and Drug Administration
FEV_1	forced expiratory volume in 1 second
FGF	fibroblast growth factor
FKBP	FK506-binding proteins
FLAP	five-lipoxygenase-activating-protein
fMLP	formyl-methionyl-leucyl-phenylalanine
FOS	fructo-oligosaccharides
FoxP3	forkhead box P3
FP	PGF receptor
FSH	follicle-stimulating hormone
5-FU	5-fluorouracil
GAGE	G antigen
GALT	gut-associated lymphoid tissue
G-CSF	granulocyte-colony stimulating factor
GH	growth hormone
GHRH	growth hormone releasing hormone
GI	gastrointestinal
GITR	glucocorticoid-induced tumor-necrosis factor receptor
GlyCAM-1	glycosylation-dependent cell adhesion molecule 1
GM-CSF	granulocyte-monocyte colony-stimulating factor
GMP	guanosine monophosphate
GOS	galacto-oligosacharides
gp	glycoprotein
GPCR	G protein-coupled receptor
GPI	glycosylphosphatidyl inositol
GPMT	guinea pig maximization test
GPx	glutathione peroxidase
GR	glucocorticoid (corticosteroid) receptor
GRE	glucocorticoid response element
GRK	G protein receptor kinase
GSH-Px	glutathione peroxidase
GTP	guanosine triphosphate
GVHD	graft-*versus*-host disease
GVT	graft-*versus*-tumor
H	histamine
H&N	squamous cell carcinoma occurring in the head and neck region
H_2O_2	hydrogen peroxide
HAART	highly active antiretroviral therapy
HAE	hereditary angio-edema
HAMA	human anti-mouse antibody
HBD	human-β defensin
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HDAC	histone deacetylase
HDL	high-density lipoprotein
HDT	high-dose chemotherapy
HER-2	human epidermal growth factor R 2

Her-2/neu	human epidermal receptor-2/neurological
HETE	hydroxyeicosatetraenoic acid
HFA	hydrofluoroalkane
HGPRT	hypoxanthine-guanine phosphoribosyl-transferase
Hib	*Haemophilus influenzae* type b
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMT	histamine *N*-methyltransferase
HMW	high molecular weight
HOCl	hypochlorous acid
HOP	HSP70/HSP90 organizing protein
HPA	hypothalamo-pituitary adrenal axis
HPETE	hydroperoxyeicosatetraenoic acids
HPG	hypothalamic-pituitary-gonadal axis
HPT	hypothalamic pituitary thyroid
HPV	human papilloma virus
HRF	homologous restriction factor
HSA	human serum albumin
HSC	hematopoietic stem cells
HSP	heat shock protein
HSR	heat shock response
5-HT	serotonin
HTL	helper T lymphocyte
i.d.	intradermal(ly)
i.m.	intramuscular(ly)
i.v.	intravenous(ly)
IAR	immediate asthma response
IBD	inflammatory bowel disease
IC	intracellular
iC3b	inactivated large fragment of complement factor 3
IC_{50}	inhibitory concentration 50% (concentration which inhibits 50% of activity)
ICAM	intercellular adhesion molecule
ICAT	isotope-coded affinity tagging
ICE	interleukin-1α converting enzyme
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
ICOS-Ligand	inducible costimulator ligand
ICS	inhaled corticosteroid
ID-2	inhibitor of differentiation-2
IDO	indoleamine 2,3 dioxygenase
IEC	intestinal epithelial cells
IEF	isoelectric focusing
IFN	interferon
IFN-γ	interferon gamma
Ig	immunoglobulin
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IGF-1	insulin-like growth factor-1
IgG	immunoglobulin G
IgG Ab	immunoglobulin G antibodies
IgM	immunoglobulin M
IgX	immunoglobulin X (X = A, D, E, G or M)
IL	interleukin
IL-1	interleukin 1
IL-1R	interleukin-1 receptor
IL-2	interleukin 2
IL-6	interleukin 6
IL-10	interleukin 10
IL-12	interleukin 12
IMPDH	inosine monophosphate dehydrogenase
INH	isoniazide
iNOS	inducible nitric oxide synthase
INR	immediate nasal response
IP	PGI_2 receptor
IP_3	inositol 1,4,5-triphosphate
IP-10	IFN-γ-induced protein 10 (CXCL10)
IPC	interferon-producing cell
IPG	immobilized pH gradient
IPV	inactivated polio vaccine
IRAK	IL-1 receptor-associated kinase
IRM	immune response modifier
IRS	insulin receptor substrates
IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
ITP	immune thrombocytopenia (formerly idiopathic thrombocytopenic purpura)
iTreg	induced T regulatory (cell)
IU	international unit
IV	intravenous
IVIG	intravenous immunoglobulin
IκB	inhibitor-κB
J	joining (gene segment)
JAK	Janus-activated kinase

JAK/STAT	Janus activated kinase/signal transducer activator transcription
JC virus	a human polyoma virus
JNK	c-jun N-terminal kinase
K_{Ca}	calcium-activated potassium channel
kDa	kilo Dalton
KIR	killer cell immunoglobulin-like receptor
L	ligand
LABA	long-acting inhaled β_2-agonists
LAD	leukocyte adhesion deficiency
LAD-1	leukocyte adhesion deficiency type-1
LAF	lymphocyte-activation factor
LAK	lymphokine-activated killer
LAMA	long-acting muscarinic antagonist
LAR	late asthma response
LBP	LPS-binding protein
LDL	low-density lipoprotein
LFA-1	leukocyte function-associated antigen-1
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
LLNA	local lymph node assay
LMP	latent membrane protein
LMW	low molecular weight
L-NAME	L-N^G-nitro-arginine methyl ester
L-NMMA	L-N^G-monomethyl arginine
LNR	late nasal response
5-LO	5'-lipoxygenase
LOX	lipoxygenase
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LT	leukotriene
LTB_4	leukotriene B_4
mAb	monoclonal antibody(ies)
Mac-1	macrophage adhesion protein-1 (CR3)
MAdCAM-1	mucosal addressin cell adhesion molecule 1
MAG	myelin-associated glycoprotein
MAGE	melanoma antigen
Mal	MyD88-adapter-like
MALDI	matrix-assisted laser desorption/ionization
MALT	mucosa-associated lymphoid tissue
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
MART	melanoma antigen recognized by T cells
MASP	MBL-associated serine protease
MBL	mannan-binding lectin
MCAT	mass-coded abundance tagging
MCP	membrane cofactor protein
MCP-1	monocyte chemotactic protein 1
M-CSF	macrophage colony-stimulating factor
MDI	metered-dose inhaler
MDP	muramyl dipeptides
MDR	multiple drug resistance
MDSC	myeloid derived suppressor cell
MG	myasthenia gravis
mHAg	minor histocompatibility antigen
MHC	major histocompatibility complex
MHLW	Ministry of Health, Labor and Welfare in Japan
MIC	minimal inhibitory concentration
mIg	membrane-bound immunoglobulin
MIMP	methyl inosine monophosphate
miRNA	microRNA
MKK	MAPK kinase kinase
MLV	multilamellar vesicles
MMN	multifocal motor neuropathy
MMP	matrix metalloproteinase
MMR	measles-mumps-rubella
MOA	mechanism of action
MODS	multiple organ dysfunction syndrome
MOG	myelin oligodendrocyte glycoprotein
MOX	monooxygenase
MPA	mycophenolic acid
MPL	monophosphoryl lipid A
MPO	myeloperoxidase
MPTP	1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MSH	melanocyte-stimulating hormone
MT	metallothionein
MTD	maximum tolerated dose
mTOR	mammalian target of rapamycin
MTP-PE	muramyl tripeptide phosphatidylethanolamide
MTX	methotrexate
MudPIT	multidimensional protein identification technology
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form

NANC	noradrenergic non-cholinergic
NDV	Newcastle disease virus
NF-κB	nuclear factor-κB
NFAT	nuclear factor of activated T cells
NGF	nerve growth factor
NHL	non-Hodgkin's lymphoma
NHS-ester	*N*-hydroxy-succinimide ester
NIEHS	U.S. National Institute of Environmental Health Sciences
NIOSH	U.S. National Institute for Occupational Safety and Health
NK (cell)	natural killer (cell)
NKA	neurokinin A
NKB	neurokinin B
NKR	natural killer receptors
NK-T	natural killer-T
NLR	NOD-like receptor
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain protein
NOX	non-phagocytic oxidase
NSAID	non-steroidal anti-inflammatory drugs
(N)SCLC	(non) small-cell lung cancer
NTP	U.S. National Toxicology Program
nTreg	natural T regulatory (cell)
NY-ESO	New York-esophagus
O_2^-	superoxide
ODN	oligodeoxynucleotide
OECD	Organization for Economic Co-operation and Development
OID	optimal immunomodulatory dose
oligo dT	oligonucleotide comprising several thymidines
$ONOO^-$	peroxynitrite
OPV	oral polio vaccine
OS	overall survival
p53	protein-53
pAb	polyclonal antibody(ies)
PAF	platelet-activating factor
PALE	post-antibiotic leukocyte enhancement
PALS	periarteriolar lymphoid sheath of spleen
PAMP	pathogen-associated molecular pattern
PB	peripheral blood
PBL	peripheral blood leukocyte
PBMCs	peripheral blood mononuclear cells
PBP	penicillin-binding protein
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PE	phycoerythrin
PECAM-1	platelet-endothelial-cell adhesion molecule 1
PEG	polyethylene glycol
PerCP	peridinin chlorophyll protein
PFN	perforin
PFS	progression-free survival
PGE_2	prostaglandin E_2
PGI_2	prostacyclin
PGN	peptidoglycan
P-gP	P-glycoprotein
PGs	prostaglandins
PHA	phytohemagglutinin
p*I*	isoelectric point
PI3 kinase	phosphatidylinositol-3-kinase
PID	primary immunodeficiency
PigR	polymeric Ig receptor
PIP2	phosphatidyl-4,5-inositol bisphosphate
PIP3	phosphatidylinositol (3,4,5) trisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC-α	protein kinase C-α
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PLNA	popliteal lymph node assay
PMA	phorbol myristate acetate
PMBC	peripheral mononuclear blood cells
pMDI	pressurised metered dose inhaler
PMF	peptide mass fingerprinting
PML-RARα	promyelocytic leukemia-retinoic acid receptor α
PMN	polymorphonuclear cell (leukocyte/neutrophil)
PNH	paroxysmal nocturnal hemoglobinurea
PNS	peripheral nervous system
POMC	proopiomelanocortin
PPH	phosphatidate phosphohydrolase
PRA	panel-reactive antibody

PRL	prolactin
PrP^{Sc}	misfolded prion protein
PRR	pattern recognition receptor
PSA	prostate-specific antigen
PSCT	peripheral stem cell transplant
PSGL-1	P-selectin glycoprotein ligand-1
PSMA	prostate-specific membrane antigen
PTCA	percutaneous transluminal coronary angioplasty
PTK	protein tyrosine kinase
PTM	post-translational modification
PUFAs	polyunsaturated fatty acids
Px	peroxidase
Qdot	quantum dot
QOL	quality of life
QSAR	quantitative structure-activity relationship
QTOF	quadrupole time-of-flight
r.f.	radio frequency
RA	rheumatoid arthritis
RAg	reporter antigen
RAG	recombinant activation gene protein
RAGE	receptor for advanced glycation end products
RANTES	regulated on activation, normal T cell expressed and secreted
RAg-PLNA	reporter antigen popliteal lymph node assay
RBL	rat basophil leukemia
RDA	recommended daily allowance
RFS	recurrence-free survival
RhD	Rhesus D
RIG	retinoid acid inducible gene
RIVM	Rijks Institute voor Volksgezondheit en Milieu (Dutch National Institute for Public Health and the Environment)
RNA	ribonucleic acid
RNCl	secondary *N*-chloramine
ROS	reactive oxygen species
rRNA	ribosomal RNA
RSV	respiratory syncytial virus
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
S	Svedberg coefficient or chemical symbol for sulfur
SAP	serum amyloid protein
SAPHO	synovitis acne pustulosis hyperostosis osteitis
SAR	structure-activity relationship
SARS	severe acute respiratory syndrome
s.c.	subcutaneous
SCF	stem cell factor
SCID	severe combined immunodeficient (mouse)
SCIG	subcutaneous immunoglobulin
SCT	stem cell transplantation
SD	standard deviation or solvent-detergent
SDS-PAGE	sodium dodecyl sulfate polyacryl amide gel electrophoresis
Sel P	selenoprotein P
SEREX	serological identification of antigens by recombinant expression cloning
SG	serglycine
SH2	src-homology 2
SHIP	SH2-containing inositol phosphatase
SHP	SH2-containing protein tyrosine phosphatase
SIgA	secretory IgA
Siglec	sialic acid binding Ig-like lectins
sIgX	surface immunoglobulin of class X (for X *see* IgX)
SIRP	signal regulatory protein
SIRS	systemic inflammatory response syndrome
SJS	Stevens-Johnson syndrome
SLE	systemic lupus erythematosus
SLP	S-layer protein
SMAC	supramolecular activation complexes
SMX	sulfamethoxazole
SOD	superoxide dismutase
SOM	somatostatin
SP	substance P
SRBC	sheep red blood cells
SRS-A	slow-reacting substance of anaphylaxis
STAT	signal transducers and activators of transcription
STZ	streptozotocin
T-α1	thymosin-α1
TAA	tumor-associated antigen
TAC	total antioxidant capacity

TACE	TNF-α converting enzyme
TACI	transmembrane activator and calcium-modulating cyclophilin ligand interactor
TAP	transporter associated with antigen processing
TB	tuberculosis
TCC	terminal complement complex
TCD	T cell-depleted
TCGF	T cell growth factor
TCR	T cell receptor
TDI	toluene diisocyanate
TdT	terminal deoxynucleotidyl transferase
TEN	toxic epidermal necrolysis
TGF	transforming growth factor
TGF-β	transforming growth factor-β
Th	T helper cell
tiff	tagged image file format
TIL	tumor-infiltrating lymphocyte
TIR	Toll/IL-1 receptor
TIRAP	TIR adapter protein
TLR	toll-like receptor
TLRL	toll-like receptor ligand
TMP	trimethoprim
TNF	tumor necrosis factor
TNFR	TNF receptor
TNFSFL	TNF-super family ligand
TOF	time-of-flight
Tollip	Toll/IL-1R-interacting protein
TP	TXA_2 receptor
TPEN	*N*,*N*,*N*,*N*-teterakis (2-pyridilmethyl) ethylenediamine
T-PLL	T-prolymphocytic leukemia
TR	thioredoxin reductase
Tr1	T regulatory cell type 1
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TREC	TCR excision circle
Treg	regulatory T cell
TRH	thyrotropin-releasing hormone
TRIF	TIR domain-containing adapter inducing IFN-β
TRPV1	transient receptor potential cation channel subfamily V1 member 1
TSH	thyrotropin
TX	thromboxane
UMP	uridine monophosphate
UNG	uracil-*N*-glycosylase
V	variable (gene segment)
V/Q	ventilation-perfusion
VCAM	vascular cell adhesion molecule
VE-cadherin	vascular endothelium cadherin
VEGF	vascular endothelial cell growth factor
VIP	vasoactive intestinal polypeptide
VLA	very late antigen
WBC	white blood cells
WHO	World Health Organization
XLA	X-linked agammaglobulinemia
ZAP	zeta chain-associated protein 70

Introduction: Altering the chain of command in host defence

Michael J. Parnham and Frans P. Nijkamp

Defence against invasion is an essential requirement for all living organisms. Several protective mechanisms are shared across vertebrates and invertebrates and form the basic components of inborn or INNATE IMMUNITY (chapter A6). These include PHAGOCYTOSIS or engulfment of foreign particles (particularly by the LEUKOCYTES or white blood cells, the infantry of the host defence system) and also recognition of microbial components by pattern response molecules, such as TOLL-LIKE RECEPTORS, which have their counterpart in fruit flies as a mechanism of defence against fungal attack. Such non-specific responses to invasion muster cellular and humoral defences in a co-ordinated attack strategy that we recognise as INFLAMMATION.

Inflammation and host defence

The cardinal signs of INFLAMMATION are redness, swelling, pain and loss of function, which provide unmistakable evidence of the battle taking place. This process represents the FIRST LINE OF DEFENCE. Initially, when a tissue is injured or invaded by an infectious pathogen, blood coagulation and thrombosis regulate bleeding and trap LEUKOCYTES. Local hormones cause the local blood vessels to dilate and increase the flow of blood as well as enhancing the supply of "reinforcements". The endothelium of the vessel wall becomes leaky, providing the opportunity for activated blood proteins and cells to invade the damaged tissue. Protein mediators, the CYTOKINES and CHEMOKINES (chapter A5), then activate the LEUKOCYTES, guiding them along concentration gradients and ADHESION MOLECULE "stepping stones" into the battle zone. Here the various mediators (chapter A7) stimulate the cells to release microbe-killing constituents, such as REACTIVE OXYGEN SPECIES or LYSOZYME and specific CYTOKINES (chapter A5), which orchestrate the specific populations of LEUKOCYTES needed to deal with the inflammatory agent (Fig. 1). The COMPLEMENT SYSTEM in the blood also generates proteins that attract LEUKOCYTES to the inflamed site (chapter A6), while IMMUNOGLOBULINS coat foreign particles to make them more easily subject to PHAGOCYTOSIS. A primary bacterial infection is a typical example of this type of acute inflammatory response (chapter A8).

In preparation for the successful defence of the tissue, already within a few hours, the "cleaning-up operation" is initiated. This is heralded by the suicide of the initial foot soldiers – by APOPTOSIS or programmed cell death of the neutrophilic LEUKOCYTES. These cell carcasses and other debris deposited during the battle are engulfed by mononuclear PHAGOCYTES, including MACROPHAGES, which also release repair products to stimulate regrowth of healthy tissue. Once again, local hormones and CYTOKINES (chapters A5 and A7) direct this resolution of the inflammatory process.

ADAPTIVE IMMUNITY represents a more sophisticated and finely tuned approach to defence, akin to the complex guided weaponry of modern military arsenals. The development of specific ANTIBODIES or cellular immune responses allows the organism to neutralise invading microbes or foreign particles without necessarily causing discomfort to the host. This requires prior exposure to a foreign protein, the antigen, and its uptake by LEUKOCYTES, for instance during a previous infection. The intracellular processing of ANTIGENS by antigen-presenting cells (APCs), in particular by the "professionals" – the DENDRITIC CELLS (DCs; chapter A4) in primary LYMPHOID ORGANS – is

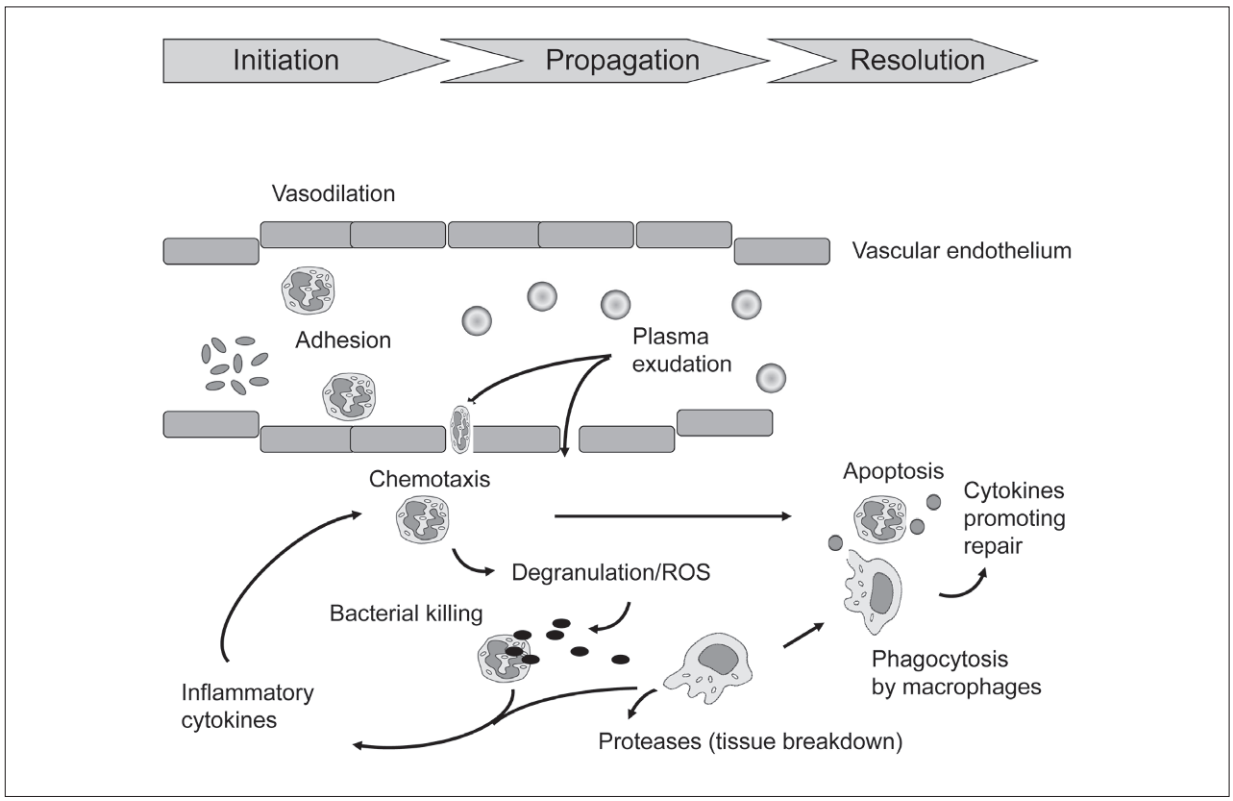

Figure 1. Simplified presentation of acute inflammation

In the presence of an inflammatory stimulus, such as bacteria or tissue injury, inflammation is initiated by damage to microvessels causing blood coagulation and platelet aggregation. Local inflammatory mediators, including eicosanoids, amines and peptide cytokines and chemokines are generated (particularly by tissue macrophages) and act on the vascular endothelium and circulating leukocytes to cause the leukocytes (initially neutrophils and potentially other populations at later time points) to adhere to the endothelium. Vasodilation also occurs. This facilitates already ongoing plasma exudation. The process is further propagated by diapedesis (transendothelial migration) of leukocytes along a concentration gradient of chemokines. Within the tissue, in contact with the bacteria or injurious stimulus, the leukocytes release bactericidal proteases and reactive oxygen species, which also cause bystander tissue injury and neutrophil death by necrosis. Tissue macrophages are activated and joined by monocytes entering from the circulation, which become macrophages. These cells then initiate the resolution phase, when neutrophils die by apoptosis. Apoptotic cells are phagocytosed by macrophages, which start to generate repair inducing products and cytokines that stimulate regrowth of surrounding tissue.

followed by presentation of the antigen to the awaiting T LYMPHOCYTES. This occurs, in association with the self-restrictive MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) molecules, on the surface of the APC. The T LYMPHOCYTES - crucial cells for the adaptive immune response – have already undergone a very rigorous selection process (chapters A1 and A2), based on genetic factors, antigenic stimuli and cellular interactions in the thymus, long before their recruitment to the defence force. Depending on the battle zone (antigen, pathogen, CYTOKINES produced etc; chapter A5), a variety of sub-types of T cells are produced,

which determine the type of defence reaction to be mounted. T helper LYMPHOCYTES then turn their attention to the B LYMPHOCYTES, which have been recruited from the BONE MARROW (chapter A1). In concert with the DCs (chapter A4) and other APCs, the T cells (within special areas of LYMPHOID ORGANS) help B cells to respond to the specific antigen and to change into plasma cells, which generate specific immunoglobulin ANTIBODIES (chapter A3). Now when a new exposure to the antigenic protein occurs, as in a renewed episode of infection (chapter A8), the ANTIBODIES bind antigen, forming immune complexes, which activate complement and stimulate PHAGOCYTES to engulf the immune complexes and destroy the antigen (including the infected cells that express the antigen on their surface).

However, these complex responses, as with military weapons, can go awry and contribute to sustained chronic inflammatory, HYPERSENSITIVITY or autoimmune responses (chapter A9), which can maim or kill the very host organism that should be protected. The inability to kill infectious pathogens, such as *Mycobacterium tuberculosis* or the persistence of an irritant, together with the inadequacy of resolution of acute INFLAMMATION, can result in prolongation of the inflammatory process. Often an antigenic stimulus may persist, possibly the result of genetic susceptibility to an imbalance in adaptive immune responses and/or viral infection (chapter A8). Alternatively, cross-reactivity between a foreign antigen and an endogenous structural molecule may result in the development of AUTOIMMUNITY, such as RHEUMATOID ARTHRITIS or MULTIPLE SCLEROSIS, in which the immune cells attack host tissues. Low level persistent INFLAMMATION is thought to be one of the underlying causes of cancer, in which cells are transformed by genetic mutations, escape surveillance by cells of the innate and adaptive IMMUNE SYSTEM and proliferate to form destructive tumours (chapter A10). Finally, immune reactions can occur to otherwise innocuous environmental particles, such as dust or pollen or even drug molecules and lead to injurious HYPERSENSITIVITY reactions (chapter A9). The IMMUNE SYSTEM does not act in isolation and other systems in the organism, including the endocrine, central and peripheral nervous systems (chapter A11), are able to modify host defence.

Measuring host defence reactions

A variety of techniques are used in the diagnosis and evaluation of immune status. In addition to microbiological assessment of pathogen involvement and the standard clinical biochemical determinations in blood and urine, the analysis of different sub-sets of white blood cells has become an essential tool. This is done by detecting cell surface molecules by FLOW CYTOMETRY (chapter B3). ANTIBODY determination allows for an assessment of prior exposure to antigen or infectious agent (chapter B1) and the harnessing of the immune response to produce radio- and enzyme-linked immunoassays has revolutionised the detection of a wide variety of molecules, well beyond those involved in host defence reactions (chapter B2). Today, methods for gene analysis, using gene arrays and polymerase chain reaction enhancement of small quantities of nucleic acids have improved immensely the specific diagnosis of disease states (chapter B4). These analytical methods are also important in determining the type of therapy that needs to be administered when host defence reactions are deranged.

Pharmacological modulation of immune responses

The goal of pharmacological intervention is to modify the chain of command in host defence reactions, to

- facilitate rapid resolution of inflammation and avoid unnecessary tissue damage
- enhance inadequate, beneficial defence reactions
- dampen overactive immune responses
- or restore balance to disturbed immune homeostasis

The inhibition of acute, non-infectious INFLAMMATION and local pain or itch is frequently achieved with non-steroidal anti-inflammatory (chapter C14) or anti-allergic drugs (chapter C3). In inflammatory

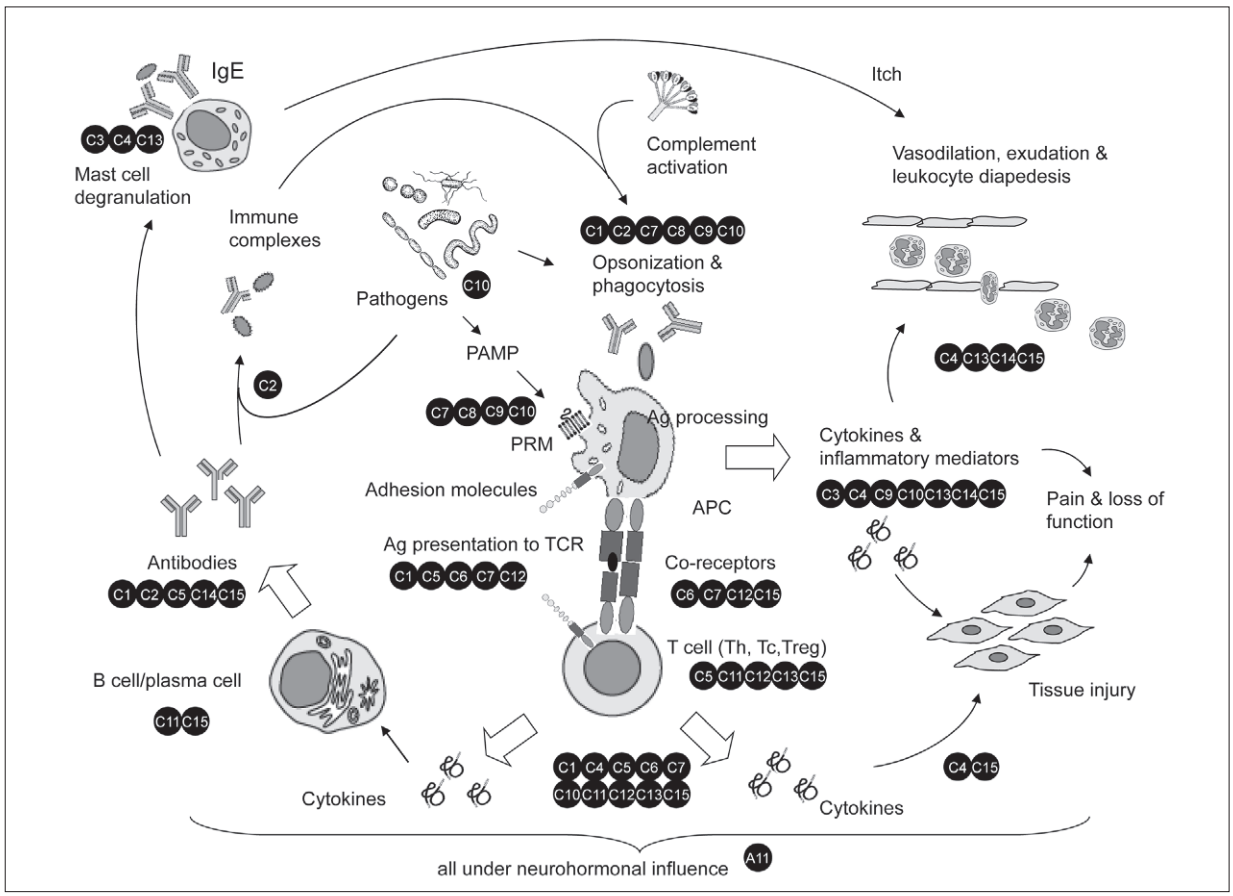

Figure 2. Summary of some of the major processes in immune defence and sites of drug action

APC, antigen-presenting cell (e.g. dendritic cell or macrophage); PAMP, pathogen-associated molecular patterns; PRM, pathogen recognition molecules. Circled numbers indicate the chapters dealing with some of the main interactions of pharmacological agents at these sites.

A11: Neuro(psycho)immunoendocrine influences; C1: Vaccines; C2: Sera and immunoglobulins; C3: Antiallergic drugs; C4: Drugs for the treatment of asthma and COPD; C5: Immunotherapy of asthma and allergy; C6: Immunostimulants in cancer therapy; C7: Immunostimulants as anti-infectives; C8: Probiotics; C9: Plant and dietary stimulants; C10: Immunomodulation by antimicrobials; C11: Cytotoxics; C12: Immunosuppressives in transplant rejection; C13: Corticosteroids; C14: Non-steroidal anti-inflammatories; C15: Disease-modifying antirheumatics.

airway diseases, in which allergic reactions are often involved, a combination of anti-inflammatory and bronchodilating drugs is required (chapter C4). In bacterial infections, additional support to endogenous host defence can be gained through the immunomodulatory properties of some antibiotics (chapter C10), while immunostimulatory agents can strengthen the IMMUNE SYSTEM in its defence against infections (chapter C7).

The most effective means to overcome attack by an infectious agent is to actively immunise the organism against the specific pathogen, using vaccines, or to administer IMMUNOGLOBULINS to provide temporary passive immunisation (chapters C1 and

C2). Similar enhancement, in this case against inadequate immune defence, is offered by immunomodulators in cancer therapy (chapter C6).

When the immune response has become excessive and pathological, a number of immunosuppressives and antirheumatic drugs are indicated, as in RHEUMATOID ARTHRITIS (chapter C15) or transplant rejection (chapter C12), while repeated antigen administration can rebalance the immune response, as in ALLERGY IMMUNOTHERAPY (chapter C5). Older, less-specific drugs, such as CYTOTOXIC agents (chapter C11), are being used much less commonly to suppress excessive immune reactions, but some long-established drugs, including acetylsalicylic acid (chapter C14) or corticosteroids (chapter C13) have never lost their therapeutic usefulness. Equally, the scientific basis for therapy with dietary and plant-derived agents – many with their roots in folklore – has become stronger in recent years (chapters C8 and C9). At the same time, the increasing use of highly specific biological agents, including MONOCLONAL ANTIBODIES and RECOMBINANT CYTOKINES, is revolutionising immunopharmacology, particularly in cancer and the treatment of AUTOIMMUNE DISEASES (chapters C6 and C15). Such BIOLOGICALS, although needing to be administered by injection because of their protein nature, have the considerable advantage that they TARGET highly specific molecules. As a result, not only are discreet pathological mechanisms attacked, but also the likelihood of adverse effects of the drugs is reduced. Undesirable effects were common among the older CYTOTOXIC, antirheumatic and anti-inflammatory agents, but the science of toxicology has also progressed and regulatory authorities governing the registration of drugs now provide clear guidance on the testing that is needed to improve the safety of new immunopharmacological agents (chapter D1).

This textbook provides the reader with an overview of all these principles of immunopharmacology. Figure 2 summarises these processes and indicates where the various classes of pharmacological agents considered in this textbook act.

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A

Immunity

A1

Hematopoiesis and lymphocyte development: An introduction

Frank J.T. Staal, Karin Pike-Overzet, Menno C. van Zelm

Introduction: Blood cell development and immunity

The production of blood and immune cells is a continuous process throughout life and essential for the existence of any individual human being. This process is referred to as HEMATOPOIESIS (from the Greek hematos, blood, and poiein, to generate) and includes the generation of ERYTHROCYTES (red cells), THROMBOCYTES (platelets) and LEUKOCYTES (white blood cells, consisting of various types of GRANULOCYTES, LYMPHOCYTES and MONOCYTES). The major site of HEMATOPOIESIS is the BONE MARROW, but it can occur at other (extramedullary) locations as well.

The human body has several ways of combating bacteria, viruses and other pathogens, as we are exposed to millions of microorganisms on a daily basis. Collectively, these potentially pathogenic microorganisms or substances in the external environment are referred to as ANTIGENS. Physical barriers, such as our epithelial layers, and physiological defense mechanisms such as the low pH in our stomach and a slightly acidic layer on our skin, form a first line of defense. When pathogens succeed in passing the physical and physiological barriers, cells of our IMMUNE SYSTEM play an important role in attacking them. MACROPHAGES and GRANULOCYTES are the main cell types involved in the first immunological response to invading pathogens. Both cell types are capable of engulfing and digesting microorganisms and are part of the INNATE IMMUNE SYSTEM (see chapter A6). In addition to being responsible for the early defense against microbes, the INNATE IMMUNE SYSTEM plays an important role in activating the adaptive IMMUNE SYSTEM.

The adaptive IMMUNE SYSTEM, also called the antigen-specific IMMUNE SYSTEM, consists of LYMPHOCYTES and ANTIBODIES and is capable of recognizing and fighting a wide range of pathogens. T and B LYMPHOCYTES make up the cellular compartment of the adaptive IMMUNE SYSTEM. These cells recognize a large variety of ANTIGENS, stretches of amino acids, polysaccharides or lipids present on or derived from pathogens. B LYMPHOCYTES produce ANTIBODIES. T LYMPHOCYTES function either as 'stimulators' in immune responses, or directly destroy infected cells, mainly virus-infected cells. In addition, the adaptive IMMUNE SYSTEM has the ability to remember and adjust to the pathogens they encounter. A compromised adaptive IMMUNE SYSTEM can lead to severe illness and death, underscoring its importance.

Since basic cellular processes are similar for HEMATOPOIESIS, including lymphocyte development and reactions of mature LYMPHOCYTES (i.e., cellular proliferation, response to CYTOKINES), pharmacological agents affecting mature LYMPHOCYTES can also affect the development and maturation of these cells. For example, immunosuppressants with an antiproliferative mode of action can show BONE MARROW depression as an adverse side effect.

Hematopoietic stem cells

The STEM CELLS that give rise to all blood lineages are known as HEMATOPOIETIC STEM CELLS (HSC) and mainly reside in the BONE MARROW. Figure 1 shows a schematic overview of HEMATOPOIESIS. It is estimated that about 1 in 100 000 cells in the BONE MARROW is a true HSC [1]. Despite their low frequency, HSC have the ability to produce high numbers of new blood cells each day. The potential to sustain the supply of blood throughout an individual's lifespan can be attributed to two features that characterize HSC: self-renewal

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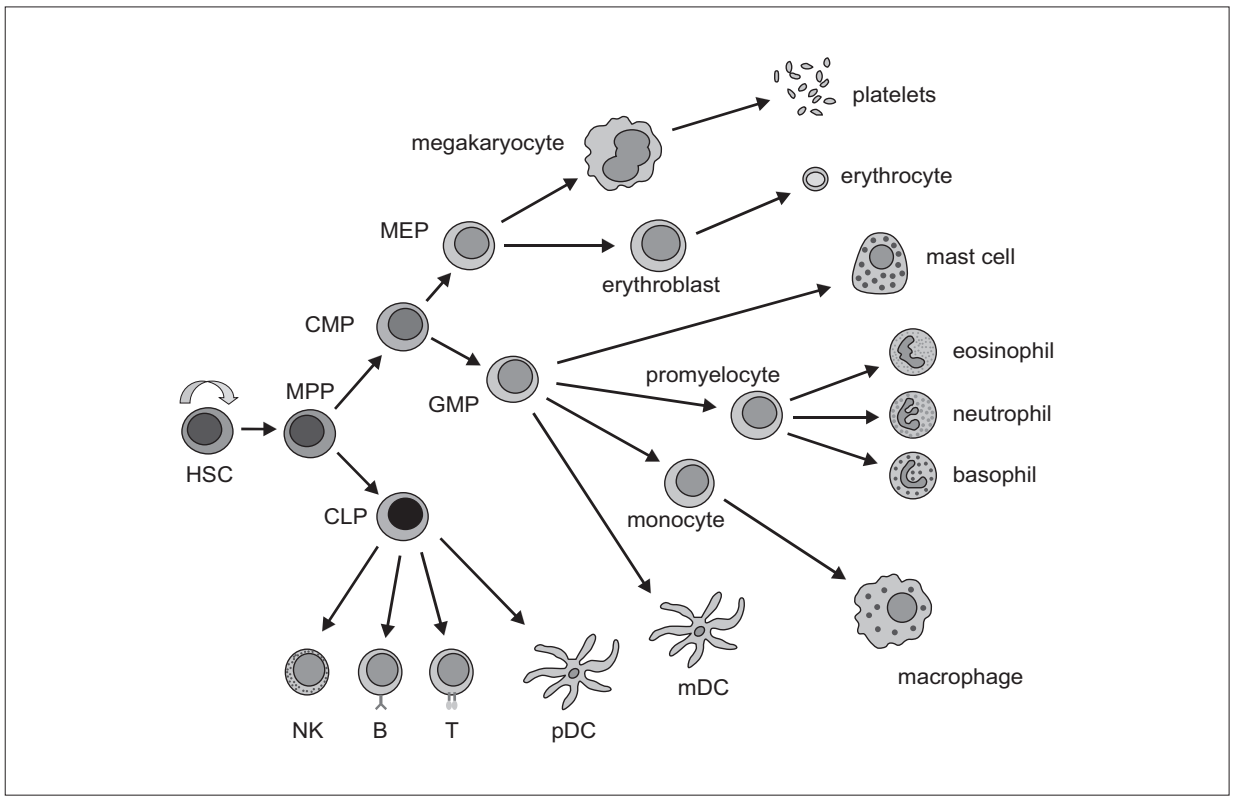

Figure 1. Schematic overview of hematopoiesis

Hematopoietic stem cells (HSC) are responsible for blood cell production throughout the lifetime of an individual. Pluripotent HSC can give rise to several different hematopoietic lineages while retaining the capacity for self-renewal. Lineage-committed progenitor cells produce progeny destined to differentiate into red cells, granulocytes, lymphoid cells, and platelets.

and multipotency. Self-renewal of HSC is defined as the ability to divide while retaining undifferentiated features. The multipotency of HSC refers to their capacity to differentiate into multiple different cell types, in this case all types of blood cells. The multilineage differentiation capacity of HSC was initially demonstrated by the prevention, through injection of BONE MARROW cells, from hematopoietic failure after total-body irradiation of mice [1]. HSC are cells with a slow division rate. Up to 20% of all divisions are asymmetrical, resulting in one daughter cell that retains stem cell features and other daughter cells that differentiate [2–4]. It is difficult to identify HSC by their size and shape, since their morphology and behavior in culture resembles that of white blood cells. The development of MONOCLONAL ANTIBODIES recognizing cell surface markers, as well as fluorescence-activated cell sorting (FACS; see chapter B3) has facilitated the purification of small subsets of cells. In combination with *in vivo* and *in vitro* assays, populations that contain cells with HSC capacities have been identified. Besides being present in BONE MARROW, human HSC can also be isolated from umbilical cord blood, and, when mobilized by granulocyte-colony stimulating factor (G-CSF), from peripheral blood [5,6]. This is an important pharmacological means to manipulate the numbers of STEM CELLS that can be used for clinical transplantation applications.

Murine HSC are characterized by the surface expression of Sca-1, C-kit, and CD38, low expression levels of the Thy-1, low to absent CD34, and the lack of lineage markers (B220, Mac-1, Gr-1, CD3, CD4, CD8 and Ter119). The most widely used HSC population in the mouse is the so-called LSK population: lineage marker negative, Sca-1$^+$ and C-kit$^+$. Within this population two subsets can be distinguished, namely, long-term [7–10] and short-term [8]. For both mouse and man, other markers are being continuously evaluated and added to diagnostic methods in an attempt to more precisely define true HSC. Of note are the so-called SLAM markers, CD50 and CD48, which further subdivide the LSK population into cells enriched for long-term or short-term repopulating STEM CELLS and multipotent progenitors [11, 12].

While blood cells in the embryo may first be derived from hematopoietic cells residing in the yolk sac, current evidence strongly suggests that the aorta-gonad-mesonephros (AGM) region is the source of the definitive adult hematopoietic system, which subsequently colonizes the liver and then the BONE MARROW. Thus, during fetal life the liver is an important hematopoietic organ in which HSC also expand in numbers, whereas in adults, HSC occur almost uniquely in the BONE MARROW [13].

Because they can give sustained reconstitution of all blood lineages, transfer of HSC has been used in numerous therapeutic protocols. Conditions that are regularly treated by HSC transfer include leukemia, lymphoma, different types of inherited anemia, inborn metabolic disorders, Wiskott-Aldrich syndrome, and severe combined immunodeficiency (SCID) [14–19].

Lymphocyte development

T cells, B cells and NATURAL killer (NK) cells are the three major types of cells that can be distinguished within the lymphocyte lineage. NK cells are part of the INNATE IMMUNE SYSTEM and play a key role in the host defense against virally infected cells as well as tumors. Upon activation of NK cells, granules that contain CYTOTOXIC proteins are released from their cytoplasm, resulting in the destruction of the TARGET cell. T and B LYMPHOCYTES make up the antigen-specific adaptive IMMUNE SYSTEM and are both able to recognize a broad spectrum of ANTIGENS. T cells bear T CELL RECEPTORS (TCR) on their cell surface that are capable of recognizing unique ANTIGENS when presented in the context of MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) molecules by cells of the INNATE IMMUNE SYSTEM or by B cells (see chapter A2). Similar to T cells, B LYMPHOCYTES express specialized antigen RECEPTORS, called B cell antigen RECEPTORS or IMMUNOGLOBULINS (Ig), with a single antigenic SPECIFICITY. These Ig are membrane bound but can also be secreted when B cells mature into plasma cells. The secreted form of B cell RECEPTORS are called ANTIBODIES, and are part of the humoral IMMUNE SYSTEM (see chapter A3). Like all blood cells, LYMPHOCYTES are highly specialized cells that develop from a common stem cell through a process called lymphopoiesis [20, 21]. Key decisions made during the multistep process of lymphopoiesis depend on a large number of signals conveyed by cell-cell interactions, soluble factors, and the extracellular matrix within stromal microenvironments at specialized sites of maturation.

Specificity: Rearrangement of genes encoding antigen receptors

For HSC to become specialized B and T LYMPHOCYTES, the cells have to undergo several developmental steps. A common feature of T and B cell development is the process of gene rearrangement of TCR and Ig loci, respectively. This highly specialized process of cutting and pasting gene segments within the Ig and TCR loci, also called V(D)J RECOMBINATION, assures the generation of antigen RECEPTORS with an extensive REPERTOIRE [22]. Antigen RECEPTORS are made up of constant and variable domains. The constant domains are equipped to provide structure, effector function, and signaling of antigen RECEPTORS, while the variable domains are responsible for specific antigen recognition [23, 24]. If each unique antigen RECEPTOR were coded by a separate gene, the size of the human genome would not suffice. Therefore, the exon coding for the variable domain of Ig and TCR proteins is generated by more or less random RECOMBINATION of

variable (V), diversity (D), and joining (J) gene segments. The Ig heavy chain locus consists of V, D and J segments, whereas kappa and lambda light chain loci only have V and J segments. Similarly, the TCRA and TCRG loci are composed of V and J segments, while the TCRB and TCRD loci contain V, D and J segments. In loci containing V, D and J segments, the D to J rearrangement takes place first, followed by V to DJ rearrangements. An example of V(D)J RECOMBINATION is shown in Figure 2. The initiation of RECOMBINATION is directed by RECOMBINATION signal sequences (RSSs) that flank the coding gene segments. RSSs are built up of two conserved sequences, a heptamer (conserved 7 base pair sequence) and a nonamer (conserved 9 base pair sequence), separated by a non-conserved spacer sequence of 12 or 23 base pairs. The consensus heptamer sequence is CACAGTG and the nonameric consensus sequence is ACAAAAACC. Generally, RECOMBINATION occurs between an RSS with a 12-base pair spacer and an RSS with a 23-base pair spacer, the so-called "12/23 rule". Two proteins, recombinase activating gene (RAG) 1 and 2, bind to an RSS as a complex. RAG1 and RAG2 proteins are indispensable for initiation of V(D)J RECOMBINATION and ultimately generate the cleavage by which the interspersed DNA is cleaved out, leaving an excision circle [25]. Other modes of RECOMBINATION, such as inversions and deletions also are possible.

In summary, Ig and TCR gene segments are ligated through V(D)J RECOMBINATION creating RECEPTORS that can recognize a multitude of ANTIGENS. During this process junctional diversity is introduced by excision and addition of nucleotides. The stretch of DNA located between the rearranged segments is excised from the genome resulting in a circular excision product bearing the signal joint.

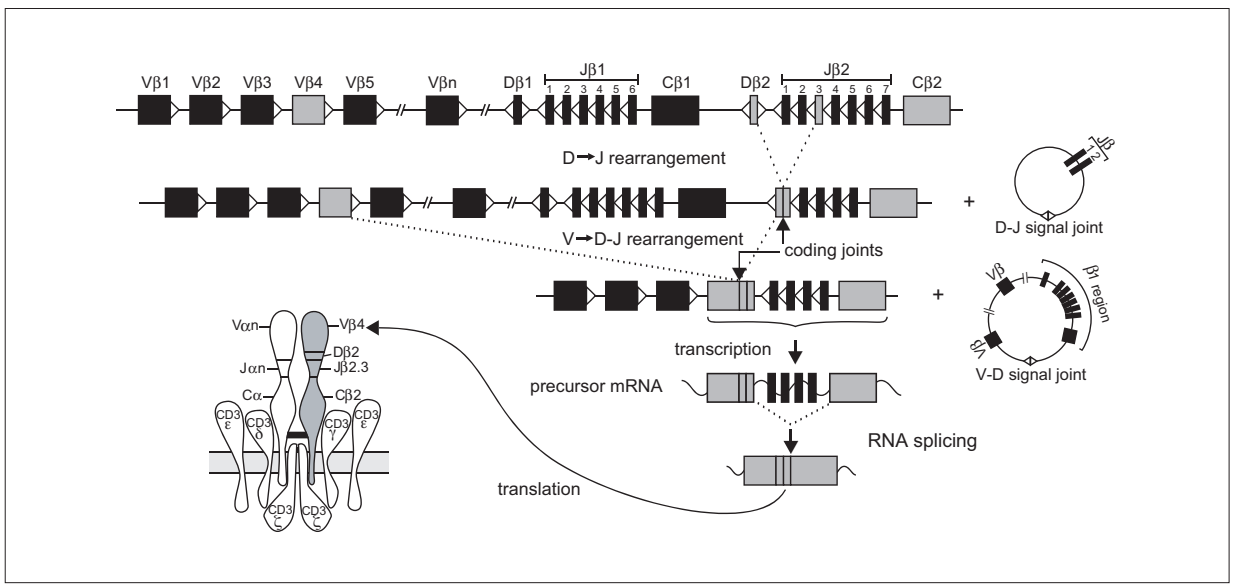

FIGURE 2. SCHEMATIC DIAGRAM OF SEQUENTIAL REARRANGEMENT STEPS, TRANSCRIPTION, AND TRANSLATION OF THE *TCRB* GENE DURING T CELL DIFFERENTIATION

In this example, first a Dβ2 to Jβ2.3 rearrangement occurs, followed by Vβ4 to Dβ2-Jβ2.3 rearrangement, resulting in the formation of a Vβ4Dβ2Jβ2.3 coding joint. The two extrachromosomal TCR excision circles (TRECs) that are formed during this recombination process are also indicated; they contain the D-J signal joint and V-D signal joint, respectively. The rearranged TCRB *gene is transcribed into precursor mRNA, spliced into mature mRNA, and finally translated into a TCRβ protein. The mature TCR, a dimer of a TCRα and a TCRβ chain, is expressed on the surface membrane together with the CD3 complex, consisting of CD3γ, CD3δ, CD3ε and CD3ζ proteins.*

T cell development

T cell development is a highly regulated, multi-step process aimed at generating mature, functional T cells bearing TCRs that are capable of recognizing a broad range of ANTIGENS in the context of self-MHC. In contrast to all other hematopoietic lineages that develop in the specialized microenvironment of the BONE MARROW, development of T cells from pluripotent HSC takes place in the thymus. Throughout life, T cell precursors from the BONE MARROW seed the thymus and differentiate into T cells. Mature T cells express a heterodimeric TCR that is either composed of one α chain and one β chain or one γ chain and one δ chain. When progressing through T cell development, cells undergo lineage commitment, TCR gene rearrangements, proliferation and selection.

Using cell surface markers, several T cell developmental stages can be distinguished (Fig. 3). Primarily, thymocytes are subdivided into double-negative (DN), double-positive (DP) and single-positive (SP) populations, referring to the expression of the co-RECEPTORS CD4 and CD8. The most immature thymocytes lack expression of both CD4 and CD8 and are therefore called DN. In mouse and humans, additional but different surface markers are used to further subdivide the DN stage. For mouse, the markers CD25 and CD44 are used: $CD44^+CD25^-$ cells are called DN1 cells, $CD44^+CD25^+$ cells are referred to as DN2, DN3 cells express CD25 but no CD44, and DN4 cells express neither CD25 nor CD44. The most immature human thymocyte population is characterized by the expression of CD34, but lacks CD1a and CD38 expression, and resembles the murine DN1 population [26–32]. The next stage of

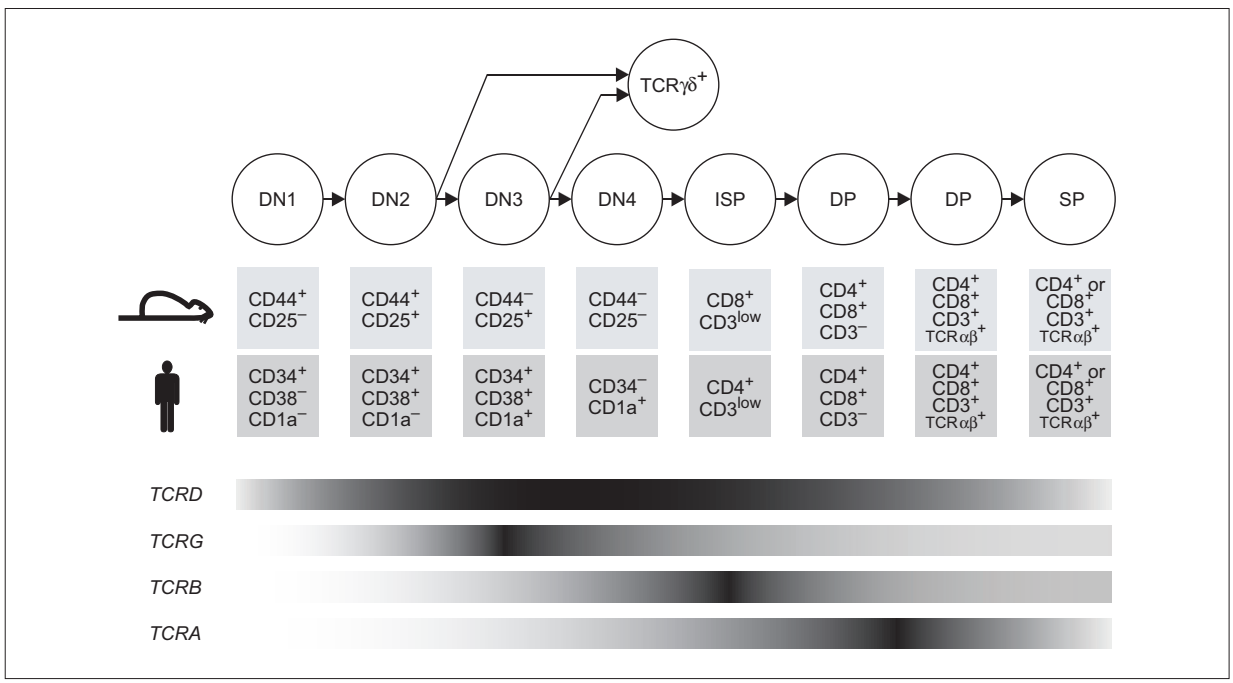

FIGURE 3. T CELL DEVELOPMENT

Consecutive stages of development are described for mouse and human according to the surface markers that are expressed. Based on a previously presented model, stages of development that are comparable between mouse and man are grouped together. In the lower part of the figure, rearrangement activity of the T cell receptor loci is depicted. Light shades represent low rearrangement activity and dark shades represent high rearrangement activity.

differentiation is marked by the expression of both CD34 and CD38 and resembles the murine DN2 stage. The most mature human DN stage that can be discerned is made up of cells expressing CD34 and CD38 as well as CD1a. Thymocytes undergo a substantial number of cell divisions, six to ten, in the first DN stages [33]. The first round of gene rearrangement takes place in DN thymocytes and rearrangements are initially detected at the TCRD locus, mainly during DN2 and DN3 stages of development [24, 34]. Subsequently, TCRG rearrangements occur, mostly at the DN3 stage. When a TCRγδ is successfully formed, the developmental path of γδ T cells diverges from αβ T cell development, most likely at the DN3 stage [35]. If rearrangements at the TCRD locus are non-functional, rearrangement of the TCRB locus will proceed. Successful rearrangement of TCRB gene segments is tested by expression of the TCRβ-chain on the cell surface paired with the invariant pre-Tα RECEPTOR in the pre-TCR complex. This process is also referred to as β-selection, a major checkpoint during T cell development. Signaling through the pre-TCR will result in entry of the cell cycle.

During proliferation cells go through immunophenotypic changes: immature single-positive (ISP) cells arise when thymocytes express a CO-RECEPTOR in the absence of high levels of CD3. In humans, ISP cells express CO-RECEPTOR CD4, whereas in most strains of mice they express CD8. Subsequently, both CD4 and CD8 are expressed and therefore these cells are referred to as double positive. In the DP stage, TCRA gene rearrangements are initiated. The DP stage makes up approximately 85% of all thymocytes. After TCRA rearrangement, a TCRαβ heterodimer is expressed on the cell surface. This unique TCR is then tested for the recognition of self-MHC molecules in a process called positive selection, while negative selection tests for the absence of self-reactivity. After failing the selection criteria, approximately 95% of thymocytes die through the induction of APOPTOSIS [36]. After successfully undergoing positive and negative selection processes, thymocytes that express a functional TCR commit to either the $CD4^+$ T helper lineage or the $CD8^+$ CYTOTOXIC T lineage, ready to migrate to the periphery.

B cell development

Precursor-B cells undergo stepwise differentiation in the BONE MARROW, during which each developing B cell creates a unique antigen RECEPTOR by V(D)J RECOMBINATION in a similar fashion as to precursor-T cells in the thymus. The B cell antigen RECEPTOR (BCR) consists of two identical copies of the Ig heavy chain (IgH) and two identical copies of the Ig light chain (Igκ or Igλ), and is expressed on the membrane with CD79a and CD79b. In contrast to the TCR, the BCR can recognize complete, unprocessed antigen without the need for presentation by other immune cells.

During precursor-B cell differentiation, five functionally different stages can be identified based on the stepwise rearrangement of IgH and Ig light chains. The five precursor-B cell subsets are defined by cytoplasmic and membrane Ig expression, but can also be identified unique combinations cell surface markers (Fig. 4) [37, 38]. Prior to commitment to the B cell lineage, a stem cell is first restricted to the lymphoid lineages. This step is succeeded by specification to the B cell lineage. At this stage, the cells are identified as pro-B cells and have started V(D)J RECOMBINATION of the *IGH* loci with incomplete D_H–J_H gene rearrangements. E2A and EBF are important transcription factors that promote B cell specification, inhibit other cell fates and activate RAG gene transcription [39]. Subsequently, E2A and EBF induce transcription of Pax-5, which commits the precursor cell to the B cell lineage [40]. Pax-5 induces complete V_H–J_H gene rearrangements and the transcription of multiple B cell-specific proteins, such as CD79a, BLNK and CD19 [41]. These early committed precursor-B cells are identified by membrane CD19 expression and named pre-B-I.

Upon successful RECOMBINATION of one of the *IGH* loci, the VDJ_H exon is spliced to the μ constant exons, producing Igμ heavy chain protein. In absence of a functional Ig light chain, Igμ is expressed on the membrane with CD79a, CD79b and two proteins that form a SURROGATE light chain: λ14.1 (named λ5 in mice) and VpreB. This complex is called the pre-BCR and although it is expressed on the cell membrane, it is difficult to detect. Still, Igμ expression can

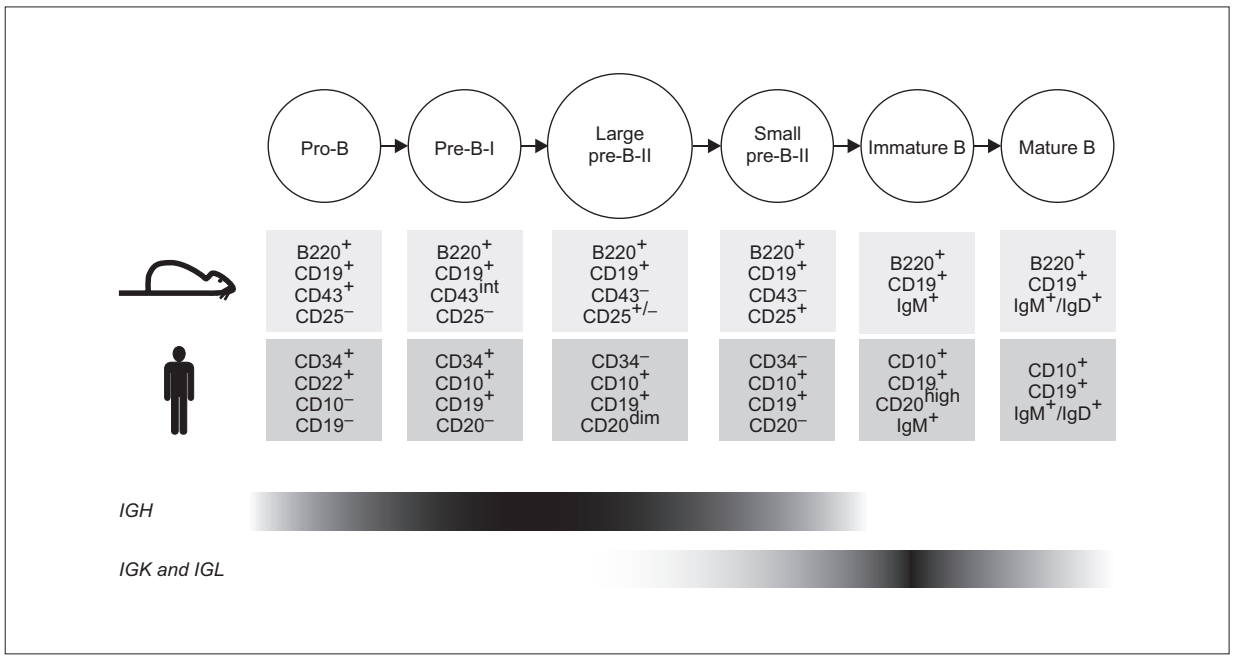

Figure 4. B cell development in the bone marrow

Consecutive stages of development are described for mouse and human according to the surface markers that are expressed. Stages of development that are comparable between mouse and man are grouped together. In the lower part of the figure, rearrangement activity of the immunoglobulin loci is depicted. Light shades represent low rearrangement activity and dark shades represent high rearrangement activity.

be detected intracellularly in all precursor-B cells that have generated a functional Ig heavy chain. Cytoplasmic Igμ$^+$ cells are defined as pre-B-II.

Directly upon membrane expression, the pre-BCR initiates multiple processes *via* signaling cascades [42]. Despite some controversy about the need for a LIGAND, it appears that pre-BCR activation is dependent on stromal cell-derived galectin-1 and the adhesion proteins (INTEGRINS) [43]. Upon activation, the kinase Lyn is recruited and signals for induction of proliferation (large pre-B-II cells). Lyn signaling also leads to down-regulation of the Rag proteins to prevent potential further *IGH* gene rearrangements on the second allele (allelic exclusion). Finally, after several rounds of proliferation the pre-BCR is down-regulated to enable further differentiation and the induction of Ig light chain gene rearrangements. The latter two processes depend on additional signaling protein, especially BLNK and BTK. In the now small pre-B-II cells, Ig light chain Vκ–Jκ gene rearrangements are initiated first. If this leads to a functional protein that can pair with the Igμ heavy chain, the cell will express an Igκ$^+$ BCR. Unsuccessful Vκ–Jκ gene rearrangements are followed by Vλ–Jλ gene rearrangements. If these yield functional protein, the B cell will express an Igλ$^+$ BCR. Membrane expression of the BCR is readily detectible and these IgM$^+$ cells are named immature B cells [44]. At this stage, the majority of all B cells express autoreactive BCRs [45]. Autoreactive B cells are removed from the REPERTOIRE by APOPTOSIS or by generation of a new BCR by additional rearrangements that replace the Ig light chain (RECEPTOR editing). Once the cell fulfills the right criteria, it will transcribe two splice variants of the Ig heavy chain, thus co-expressing IgM and IgD BCRs and migrate to peripheral LYMPHOID ORGANS.

These recent BONE MARROW emigrants are transitional cells and once they develop into naïve mature B cells they are ready to encounter and respond to their cognate antigen.

NK cell development

The third member of the lymphoid lineage, the NK cell, plays an important role in defense against virally infected cells and tumors as well as activation of the adaptive IMMUNE SYSTEM. NK cells are large granular cells that make up 10–15% of all circulating LYMPHOCYTES and are immunophenotypically characterized as $CD3^{-}CD56^{+}$ in humans. For murine NK cells, other markers are used, namely, DX5, NKG2D and NK1.1 [46].

In comparison to B and T cell development, relatively little is known about the development of NK cells. Unlike B and T cells, development of NK cells does not involve the process of V(D)J RECOMBINATION. A significant part of NK cell development takes place in the BONE MARROW and IL-15 has been identified as a critical mediator of NK cell development, since mice that lack either IL-15 or IL-15R are NK cell deficient [47,48]. In early phases of NK cell development, factors produced by stromal cells in the BONE MARROW mediate the generation of NK precursor cells. These precursors are receptive for IL-15 and are able to develop in mature NK cells. The final NK cell differentiation step can occur in the BONE MARROW, but other sites, such as lymph nodes, could also provide factors necessary for terminal NK cell differentiation. CYTOKINES other than IL-15 have also been described to contribute to NK cell development. The most important contributors are IL-2 and IL-7. The only CYTOKINES that can support the development of NK cells *in vitro*, are IL-2, IL-15 and IL-7, although resulting NK cells are functionally and phenotypically immature [49–51]. The mature NK cell population can be subdivided into $CD3^{-}CD56^{dim}$ and $CD3^{-}CD56^{bright}$ cells [52]. $CD3^{-}CD56^{dim}$ cells express high levels of CD16 and killer immunoglobulin-like RECEPTOR (KIR). The highly CYTOTOXIC nature of these cells is underscored by their ability to mediate direct CYTOTOXICITY, ANTIBODY-dependent cellular CYTOTOXICITY (ADCC) as well as lymphokine-activated killing. In contrast, the expression of CD16 and KIR is low or absent on $CD3^{-}CD56^{bright}$ cells, and these cells have a low toxicity potential. $CD3^{-}CD56^{bright}$ cells act as immunoregulatory cells and are capable of producing large amounts of interferon-γ and IL-10. A distinct feature of mainly $CD56^{dim}$ NK cells is their ability to quickly mediate cellular toxicity. NK cell responses are regulated by a balance of signals from activating and inhibiting RECEPTORS, i.e., KIR (KILLER CELL INHIBITORY RECEPTOR). In the absence of activating RECEPTOR ligation, effector function is inhibited as long as the KIR molecules are bound to HLA class I molecules on the membrane of a TARGET cell. As soon as the activating RECEPTOR-LIGAND interaction overrides the inhibitory KIR signals, NK cells are activated. Once NK cells are activated, granules that hold several types of CYTOTOXIC proteins are released from their cytoplasm, which results in the destruction of the TARGET cell.

Immunopharmacology and hematopoiesis

Immunopharmacology mainly deals with the effects of agents on mature cells of the IMMUNE SYSTEM to treat AUTOIMMUNITY, allergies and acquired immune deficiencies. Commonly used immunosuppressive and other drugs that are aimed at targeting such aberrant immune responses can affect HEMATOPOIESIS, and therefore cause unwanted side effects. Examples include the DNA damaging or DNA synthesis blockers cyclophosphamide, mycophenolate mofetil and AZATHIOPRINE, which are DNA damaging agents ([53–57]; see chapters C12 and 13). There also are drugs aimed at mobilizing HSC from the BONE MARROW into the peripheral blood to harvest STEM CELLS for transplantation purposes or to strengthen the number of blood cell under cytostatic cancer treatment. These are formulations of G-CSF, a growth factor for GRANULOCYTES, but other GROWTH FACTORS such as stem cell factor, IL-8 and GM-CSF have mobilizing properties as well but are not commonly used due to side effects.

The majority of drugs that are toxic to the BONE MARROW are anticancer agents, but there is considerable variability in the severity of BONE MARROW

depression that they induce. The anticancer agents 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and busulfan (BU) are DNA ALKYLATING AGENTS used for CNS tumors and leukemias, respectively. They can severely compromise the BONE MARROW and resulting HEMATOPOIESIS.

Cyclophosphamide is also used in hematological tumors and is known to deplete the BONE MARROW of all but the most primitive precursors. Doxorubicin (adriamycin) is active against tumor cells through several mechanisms including inhibition of mitochondrial cytochrome C oxidase, intercalation of DNA and generation of toxic free radicals. Clinical use of doxorubicin is limited by myelosuppression and cardiotoxicity [58, 59].

Rapamycin is an immunosuppressive drug that blocks growth factor-induced cell proliferation [60, 61]. This drug does not affect the BONE MARROW very much, but has profound effects on T cell development in the thymus. The immunosuppressive drugs CICLOSPORIN and FK-506 have a more restricted mechanism of action (see chapter C13), i.e., inhibition of calcineurin A activity with subsequent blockade of intracellular signal transduction, leading to synthesis of CYTOKINES such as IL-2, an important growth factor for peripheral T cells [62]. While preventing T cell activation and thereby graft rejection in transplantation settings, these drugs have an interesting activity on T cell development as they appear to block APOPTOSIS during negative selection of T cells. This could potentially increase the incidence of autoimmune reactions.

Summary

The production of blood and immune cells is a continuous process throughout life and essential for the existence of any individual human being. This process is referred to as HEMATOPOIESIS. All blood cells develop from HSC in the BONE MARROW. Billions of different blood cells are produced daily from the HSC, including red cells, white cells and platelets. The adaptive IMMUNE SYSTEM, also called the antigen-specific IMMUNE SYSTEM, consists of LYMPHOCYTES and ANTIBODIES and is capable of recognizing and fighting a wide range of pathogens. T and B LYMPHOCYTES make up the cellular compartment of the adaptive IMMUNE SYSTEM. Both B and T LYMPHOCYTES carry specific antigen RECEPTORS that are generated by RECOMBINATION of V, D and J gene segments *via* regulated DNA excision and repair processes mediated mainly by RAG 1-2 proteins. B and T cell development are highly regulated processes taking place in BONE MARROW and thymus, respectively. Immunopharmacology mainly deals with the effects of agents on mature cells of the IMMUNE SYSTEM to treat AUTOIMMUNITY, allergies and acquired immune deficiencies. Commonly used immunosuppressive and other drugs targeting such aberrant immune responses can affect HEMATOPOIESIS and therefore cause unwanted side effects.

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Important websites

The websites of professional organizations have tutorials and general overview articles listed on their websites

http: //www.hematology.org/
http: //www.ehaweb.org/
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T cell subsets and T cell-mediated immunity

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Introduction

T CELL-MEDIATED IMMUNITY is an adaptive process of developing antigen (Ag)-specific T LYMPHOCYTES to eliminate viral, bacterial, or parasitic infections or malignant cells. T CELL-MEDIATED IMMUNITY can also involve aberrant recognition of self-Ag, leading to autoimmune inflammatory diseases. The Ag SPECIFICITY of T LYMPHOCYTES is based on recognition through the T CELL RECEPTOR (TCR) of unique antigenic peptides presented by MHC molecules on Ag-presenting cells (APC). T CELL-MEDIATED IMMUNITY is the central element of the adaptive IMMUNE SYSTEM and includes a primary response by naïve T cells, effector functions by activated T cells, and persistence of Ag-specific memory T cells. T CELL-MEDIATED IMMUNITY is part of a complex and coordinated immune response that includes other EFFECTOR CELLS such as MACROPHAGES, NATURAL KILLER CELLS, MAST CELLS, BASOPHILS, EOSINOPHILS, and NEUTROPHILS.

Biology of the T lymphocyte immune response

Each T LYMPHOCYTE expresses a unique TCR on the surface as the result of developmental selection upon maturation in the thymus (see chapter A1 on HEMATOPOIESIS). Mature T LYMPHOCYTES, known as naïve T cells, circulate through blood and the lymphatic system, and reside in secondary LYMPHOID ORGANS (Fig. 1). Naïve T cells are those that have not yet encountered foreign Ag and have not yet been activated. Antigenic peptides are presented to the naïve T LYMPHOCYTE in secondary LYMPHOID ORGANS by DENDRITIC CELLS (DC). DC are the most efficient "professional" APC since they also provide COSTIMULATORY SIGNALS for effective T cell activation. DC acquire Ag in non-lymphoid tissues throughout the body and migrate into secondary LYMPHOID ORGANS guided by inflammatory stimuli and CYTOKINES. APC generate antigenic peptides from a pathogenic agent or a self-Ag by antigen processing, and display them on the cell surface in the context of MHC molecules. The RECOMBINANT variability of individual αβTCR, on the other hand, ensures that at least a few naïve T cells will have high-AFFINITY binding to an antigenic peptide derived from virtually any pathogen. TCR engagement triggers a cascade of intracellular signaling events, resulting in activation of the naïve T cell.

The activated T cells rapidly proliferate (clonal expansion), migrate through the tissues to the sites of Ag presence, and perform effector functions such as cell-mediated CYTOTOXICITY and production of various CYTOKINES (soluble mediators of the immune response). CYTOTOXIC $CD8^+$ T cells are very effective in direct lysis of infected or malignant cells bearing the Ag, while $CD4^+$ T helper cells produce CYTOKINES that can be directly toxic to the TARGET cells or can stimulate other T cell effector functions and B cell ANTIBODY production, as well as mobilize powerful inflammatory mechanisms (Fig. 1) (see chapter A5 for cytokine review).

Most effector T cells will disappear after the antigenic agent is eliminated, although others will remain and form memory T cells. Unlike naïve T cells that live for few months or EFFECTOR CELLS that disappear at the end of the immune response, memory T cells may survive for years in LYMPHOID ORGANS and peripheral tissues. The easily activated memory T cells can perform immediate effector functions in peripheral tissues or undergo activation and clonal expansion in LYMPHOID ORGANS to mount a secondary immune response if the same Ag appears again.

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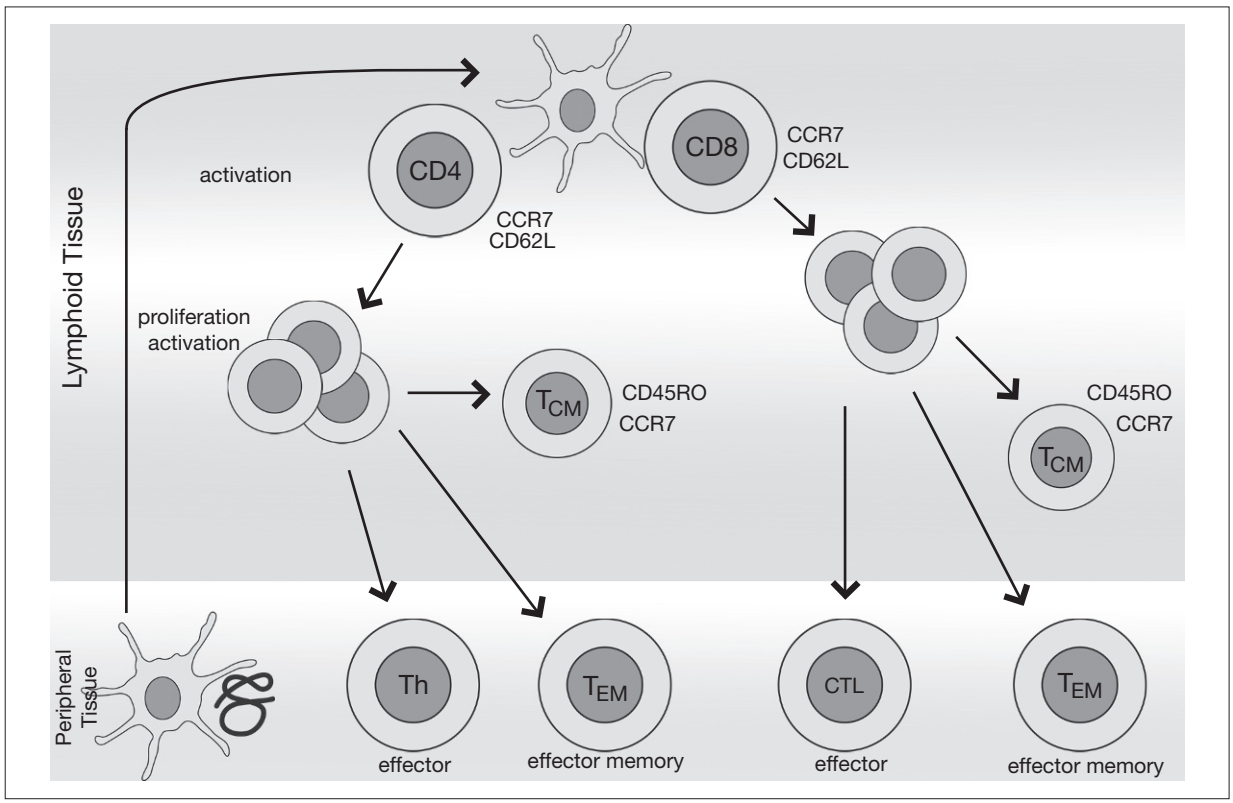

Figure 1. Development of T cell-mediated responses is a sequential process

Antigen-presenting cells (APC) can take up antigen (Ag) in peripheral tissues and migrate to secondary lymphoid tissues. Naïve T cells will be activated by recognition of MHC-peptide complexes on the APC, proliferate and differentiate into effector or memory T cells. Both CD8 (CTL) and CD4 (Th) effector T cells will migrate to peripheral tissues to exert their function. In addition, memory T cells can develop into CCR7- effector memory cells (T_{EM}) that will migrate to peripheral tissues or CCD7$^+$ central memory T cells (T_{CM}). These, in turn, can recirculate through lymphoid tissues. CCR7 is a chemokine receptor involved in T cell homing into lymphoid tissues.

Memory T cells respond much faster to the Ag than naïve T cells. Thus, in the case of infection, they help to eliminate pathogens at an early stage, thereby effectively preventing the disease spreading.

Composition of the T cell network

Lymphoid organs

The primary LYMPHOID ORGANS – the BONE MARROW and thymus – are sites of HEMATOPOIESIS and clonal selection of T cells. The T cell-mediated immune response begins in the secondary LYMPHOID ORGANS: spleen, lymph nodes, and organized lymphoid tissues associated with mucosal surfaces including PEYER'S PATCHES, tonsils, bronchial, nasal, and gut-associated lymphoid tissues. The secondary LYMPHOID ORGANS have specialized T cell-rich zones where naïve T LYMPHOCYTES are concentrated; these include the periarteriolar lymphoid sheath of the spleen (PALS) and the paracortex of the lymph nodes. Naïve T cells reside in the spleen for just a few hours and in the lymph nodes for about 1 day before they leave *via* splenic veins or

via efferent lymphatic vessels, respectively. Migrating naïve T cells eventually reach the bloodstream and soon after enter new LYMPHOID ORGANS, repeating the cycle until they become activated by antigenic peptides or die by neglect.

T cell subsets

Thymic selection results in the appearance of T cells with two types of TCR. The majority express Ag-binding αβ chains in the TCR, which are disulfide-linked heterodimers of Ig superfamily proteins (Fig. 2), forming unique structures on each T cell. αβTCR T cells have a very diverse REPERTOIRE of Ag recognition RECEPTORS and represent mature T cells that circulate through the secondary LYMPHOID ORGANS and develop adaptive immune responses. A small fraction of the T cells express γδ chains in TCR, appear to be much less heterogenic than αβTCR T cells, reside in skin and certain mucosal surfaces, and may play a role in the initial response to microbial invasion. Although the functions of γδTCR T cells are not fully understood, they are considered to be a relatively primitive part of the innate T cell response and will not be reviewed in this chapter.

αβTCR T cells are subdivided into several groups on the basis of lineage markers and functional activities. Two major surface CO-RECEPTOR molecules, CD4 and CD8, define two separate T cell lineages with different functions. $CD4^+$ cells recognize Ag in the context of MHC class II molecules (only expressed on so-called professional APC such as B cells, MACROPHAGES and DC) and produce CYTOKINES as effector T helper cells. $CD8^+$ LYMPHOCYTES are activated by antigenic peptides presented by MHC class I molecules (expressed on all nucleated cells) and form effector CYTOTOXIC T LYMPHOCYTES (CTL).

On the other hand, the functional status of the T cells allows us to distinguish naïve, effector, and memory cells, as each of these displays extensive diversity in terms of phenotype, function, and anatomic distribution. Naïve T cells are the most homogenous representatives of $CD4^+$ and $CD8^+$ subsets. Upon activation, however, they can be further distinguished by their cytokine profiles. Thus, activated $CD4^+$ T helper cells can be subdivided into Th1, Th2, Th17 and Treg

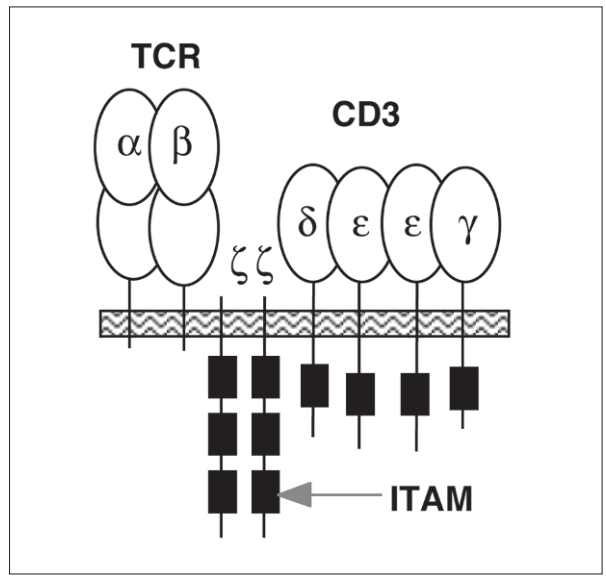

FIGURE 2
T cell receptor complex consists of αβ heterodimers responsible for antigen recognition and CD3 molecules involved in intracellular signaling. Immunoglobulin-like αβ chains are formed upon gene rearrangement and have high variability among individual T cells. Non-polymorphic CD3 chains (ζ, δ, ε, γ) contain intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) initiating cascades of signal transduction.

subsets based on production of signature CYTOKINES. In the case of the Th1/Th2 dichotomy, the characteristic CYTOKINES are: IFN-γ (Th1) *versus* IL-4, IL-5 (Th2) [1]. $CD8^+$ LYMPHOCYTES also can be assigned to Tc1 or Tc2 subsets according to their cytokine profile [2], although they do not produce the same quantities of CYTOKINES as $CD4^+$ helpers and are not efficient in B cell activation (see chapter A3). Theoretically, both effector and memory LYMPHOCYTES of $CD4^+$ and $CD8^+$ lineage can be divided into subsets based on the above criteria. In addition, there are subsets of regulatory T (Treg) cells that make T cell heterogeneity even more complex. Treg cells can be subdivided into naturally arising cells (nTreg) that are generated in the thymus, and inducible Treg (iTreg) that are converted into Treg upon activation in the periphery [3]. Many of the specific cell surface markers repre-

senting various T cell subsets can be very useful in the design of drugs for selective manipulation of the immune response.

T cell subset markers

Table 1. Phenotypic markers associated with naïve, effector or memory T cells
Many proteins are up-regulated or down-regulated rapidly after T cell activation, e.g., adhesion molecules or molecules involved in effector functions.

	naïve	effector	T_{EM}	T_{CM}
CCR7	+++	–	+/–	+++
CD62L	+++	–	+/–	+++
CD45RO	+	+++	+++	+
CD45RA	+++	–	+	++
CD95	+/–	+++	++	+/–
Granzyme B	–	+++	+/–	–
CD25	–	+	–	–
CD127	++	+/–	+	+++
CD28	++	–	+	++

Naïve T cell markers

Naïve T cells circulating in the blood, express L-selectin (CD62L), CC chemokine RECEPTOR 7 (CCR7) and leukocyte function antigen-1 (the αLβ2 integrin LFA-1). These mediate the ROLLING, adhesion, and extravasation of the cells through the high endothelial venules (specialized venules found in lymphoid tissues) in peripheral lymph nodes and mucosal LYMPHOID ORGANS.

Survival of naïve cells is maintained by low-AFFINITY TCR/self-Ag interaction and signaling as well as by the presence of IL-7. These signals are normally sufficient to maintain homeostasis of naïve T cells for several months.

Effector T cell markers

High-AFFINITY interactions of TCR with foreign antigenic peptide/MHC on mature APC following activation are reflected in phenotype changes. Activated T cells express CD69 (a very early activation antigen) and CD25 (IL-2Ra). Other important surface RECEPTORS of activated T cells are: CD40 LIGAND, which stimulates APC through binding to CD40, leading to the up-regulation of CD80 (B7-1) and CD86 (B7-2) on APC; CD28, which binds to CD80 and CD86 and propagates a costimulatory signal, thereby enhancing growth factor (IL-2) production and increasing T cell activation.

Tumor necrosis factor (TNF) RECEPTOR family molecules OX-40, CD27, and 4-1BB, also can be found on primary activated T cells. These RECEPTORS were found to sustain T cell proliferation and survival of activated T LYMPHOCYTES upon their binding to the corresponding ligands on the APC. At the peak of their proliferation, $CD4^+$ EFFECTOR CELLS were also found to change the pattern of adhesion RECEPTORS such as CD62L and sPSGL-1 (sialyated form of p-selectin glycoprotein LIGAND 1) and chemokine RECEPTOR CXCR5. $CD8^+$ CTL could also be characterized by expression of perforin and granzymes, proteins required for cytolytic functions. A particular set of surface markers may predict the homing capacity of effector T cells. For example, CXCR5 RECEPTOR helps $CD4^+$ $CD62L^-$, $sPSGL\text{-}1^-$, $CXCR5^+$ T cells to migrate into B cell-rich FOLLICLES of the lymph nodes and support ANTIBODY production. In contrast, absence of CCR7 and CD62L on CTL allows them to migrate into inflamed non-lymphoid tissues such as lung or gut and to clear pathogenic agents in these tissues.

Memory T cell markers

Memory T cells, unlike effector T cells, are not blasts nor do they enter the cell cycle. However, they are capable of circulating in lymphoid and

non-lymphoid compartments. According to the location, memory T cells are divided into central and effector memory cells and express corresponding surface markers. For example, among three phenotypes of $CD8^+$ memory cells that have been identified ($CD45RA^-$, $CCR7^+$; $CD45RA^-$, $CCR7^-$; $CD45RA^+$, $CCR7^-$), the $CCR7^+$ T cells are non-CYTOTOXIC central memory cells, while $CCR7^-$ are effector memory cells [4]. Upon contact with the appropriate Ag, effector memory cells can execute effector functions instantly, whereas central or lymphoid memory cells can rapidly proliferate, expanding and acquiring effector functions. $CD4^+$ memory cells also appear to be heterogenic. At least two subsets of $CD45RA^-$ $CD4^+$ memory cells have been identified in humans. The central memory cells express CCR7 and CD62L and reside in LYMPHOID ORGANS, producing IL-2 upon stimulation. Some of these have been found to migrate into certain INFLAMMATION sites depending on the expression of chemokine RECEPTORS such as CCR4, CCR6, and CXCR3. The other $CCR7^-$ subset with low CD62L expression produces IFN-γ and IL-4 upon stimulation and apparently represents effector memory cells.

Effectors of T cell-mediated immunity

$CD4^+$ helpers

Two major functional T helper subpopulations are distinguished by their cytokine profiles (Fig. 3). Th1 cells produce mainly IFN-γ, but also IL-2, TNF-α, and lymphotoxin. Th1 cells enhance pro-inflammatory CELL-MEDIATED IMMUNITY and were shown to induce delayed-type HYPERSENSITIVITY (DTH), B cell production of opsonizing ISOTYPES of IgG, and mediate the response to some protozoa like *Leishmania* and *Trypanosoma*. Th2 cells secrete IL-4, -5, -6, -10 and -13 and promote non-inflammatory immediate immune responses; they have been shown to be essential in B cell production of IgG, IgA, and IgE. Th1 and Th2 development routes appear to be mutually antagonistic. This has given rise to the model of polarization of immune response in accordance with the nature of the Ag and the surrounding cytokine milieu. For example, IFN-γ and IL-12 are known to support Th1 cells, while IL-4 and IL-10 assist Th2 development. Although the evidence for the polarized cytokine secretion profiles of Th1 and Th2 is indisputable, several recent studies have shown more complex patterns of cytokine interaction in different models of immune response, including autoimmune models that are inconsistent with the simple dichotomy paradigm.

Since $CD4^+$ T cells are central in the origin and regulation of AUTOIMMUNITY, emphasis has been placed on the characterization of Th subsets and their possible roles in the inflammatory process. With the discovery that the p40 subunit of the pro-inflammatory cytokine IL-12 can not only dimerize with the p35 subunit to form IL-12, but also with p19 to create IL-23, the former dogma that IL-12-driven Th1 responses were the critical contributors to INFLAMMATION had to be revised [5]. It was found that IL-23 induced production of $CD4^+$ T cells that secrete pro-inflammatory cytokine IL-17A. Subsequently, these cells were characterized as a separate Th subset, called Th17. Th17 cells are regarded as a major effector lineage with pro-inflammatory actions in diseases like RHEUMATOID ARTHRITIS, psoriasis and Crohn's disease. Contribution of Th1 cells to inflammatory diseases is still possible, although complex, given the additional regulatory contributions of IL-12 and IFN-γ in INFLAMMATION.

Th17 cells also play a prominent role in infection. In fact, Th17 is the first subset that is generated during infection. The IL-17 RECEPTOR is expressed on fibroblasts, epithelial cells and keratinocytes. Contact with IL-17 leads to production by the latter cell types of IL-6 and CHEMOKINES like CXCL8 and CXCL2 and granulocyte macrophage colony stimulating factors (GM-GSF). Altogether, this leads to recruitment of NEUTROPHILS and MACROPHAGES into the site of infection and enhances the BONE MARROW production of these cells. IL-22 produced by Th17 cells co-operates with IL-17 in the induction of ANTIMICROBIAL PEPTIDES, such as β-DEFENSINS in epidermal keratinocytes, thereby enhancing the innate acute inflammatory response in infection.

It is anticipated that a growing spectrum of Th subset lineages will be discovered, defined by the external stimuli they respond to and the transcription factors they can induce (see Fig. 3). IL-12, IFN-γ

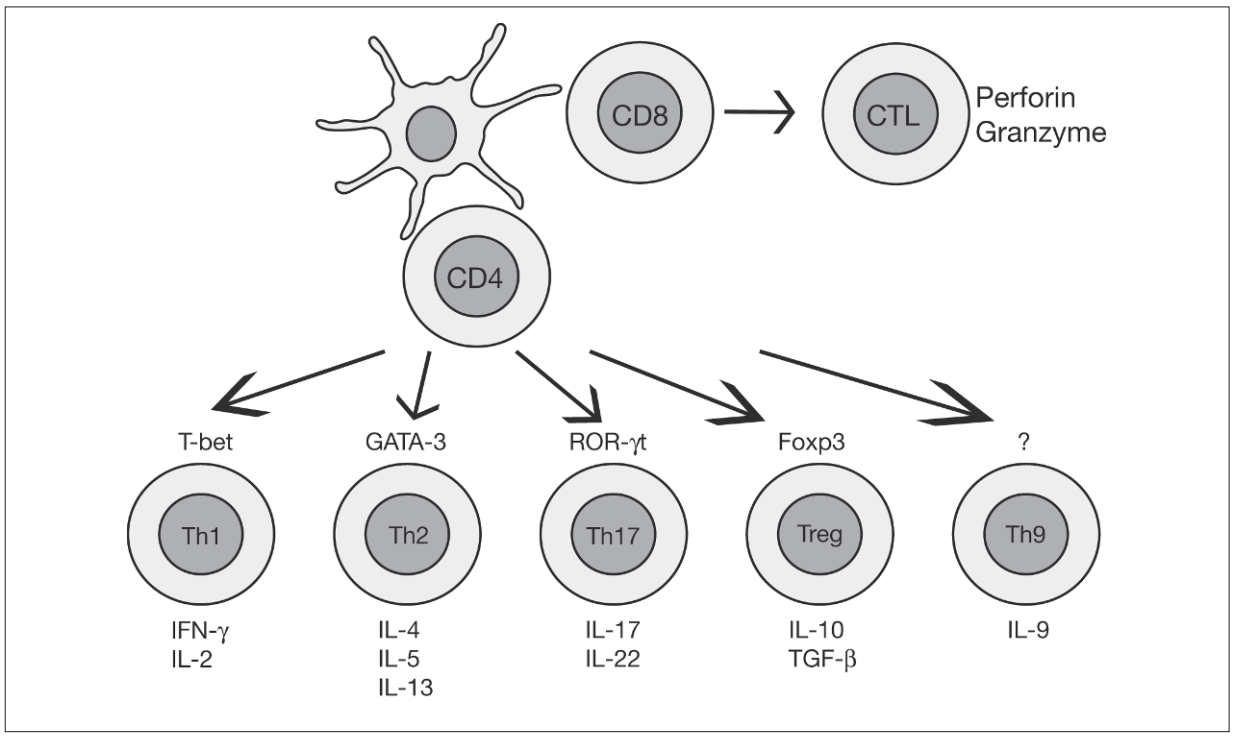

Figure 3. Differentiation of effector T cells

Ag-activated T cells will differentiate into different phenotypes depending on the cytokines in the local environment and can be characterized by their cytokine profile and by transcription factors. Th1 cells produce IFN-γ and IL-2 and express T-bet. Th2 cells produce IL-4, IL-5 and IL-13 and express GATA3. Th17 cells produce IL-17 and IL-22 and express ROR-γt. Treg can be divided into different subsets based on the expression of FoxP3 and/or the production of IL-10, TGF-β and IL-35.

and transcription factors STAT1, STAT4 and T-bet lead to the production of Th1 cells. IL-4 in combination with STAT6 and GATA-3 generates Th2 cells. Follicular T helper cells (T_{FH}) were recently defined to develop under the influence of IL-6 and transcription factor Bcl-6. Th17 cells develop in the presence of TGF-β, IL-6 and IL-23 and are characterized by the transcription factors RORγt, RORα and STAT3. Recently, Th9 cells also were proposed, a subset that develops under the influence of IL-4 and TGF-β and that produces IL-9 [6].

There are now several subsets which may have potential to produce immunological disease. Adoptive transfer of Th1 or Th17 cells produces EAE and uveitis. Colitis in mice is produced by Th1, Th2, Th17 and Th9 cells. T_{FH} can mediate the pathogenic ANTIBODY response in experimental lupus models [7].

CD8$^+$ cytotoxic T lymphocytes

CTL are derived from activated naïve CD8$^+$ cells, proliferate in the presence of IL-2, and can expand their number many thousand-fold at the peak of a primary immune response. The dramatic clonal expansion of CD8$^+$ CTL in comparison to CD4$^+$ cells can most likely be attributed to the relatively easy activation by the Ag-MHC class I complex and better survival in the circulation. Rapid expansion and the ability

of single CD8+ CTL to destroy more than one TARGET cell, while sparing "innocent" bystanders, make CTL very efficient Ag-specific EFFECTOR CELLS. Destruction of selected cells by CTL requires the establishment of cell contact with the TARGET cell and Ag recognition, thus initiating the release of cytolytic granules into the immunological synapse. CTL, unlike naïve T cells, do not require COSTIMULATORY SIGNALS upon Ag recognition in order to kill. Therefore, they can destroy a variety of TARGET cells bearing "foreign" Ag.

Mechanisms of cell-mediated cytotoxicity

Two major pathways of CYTOTOXICITY have been described in CTL: Ca^{2+}-dependent perforin/granzyme-mediated APOPTOSIS, and Ca^{2+}-independent Fas LIGAND/Fas-mediated APOPTOSIS (Fig. 4). Both pathways are initiated *via* TCR signaling. Lytic granules [secretory lysosomes containing granzymes, perforin (PFN) and the proteoglycan serglycin (SG)] [8] appear to be transported into TARGET cells as one complex. Granzymes are effector molecules capable of inducing APOPTOSIS in TARGET cells *via* caspase-dependent and -independent mechanisms. Granzymes enter into the TARGET cell directly *via* plasma membrane pores formed by PFN or *via* RECEPTOR-mediated endocytosis. In the latter case, PFN mediates the translocation of granzymes from endocytic vesicles into the cytosol. Proteoglycan SG presumably serves as a chaperone of PFN until the complex reaches the plasma membrane of the TARGET cells. Lytic granules represent a very efficient NATURAL drug delivery system.

Fas-mediated APOPTOSIS is initiated by binding of Fas molecules to the TARGET cell *via* Fas LIGAND on the CTL. The Fas molecule is a member of the TNF RECEPTOR superfamily with an intracellular "death" domain initiating caspase-dependent APOPTOSIS upon binding to Fas LIGAND. TCR cross-linking was shown to induce up-regulation of Fas LIGAND expression on the cell surface of CTL and in cytolytic granules. Fas-mediated APOPTOSIS appears to be a general phenomenon not restricted to CTL. It was found to be

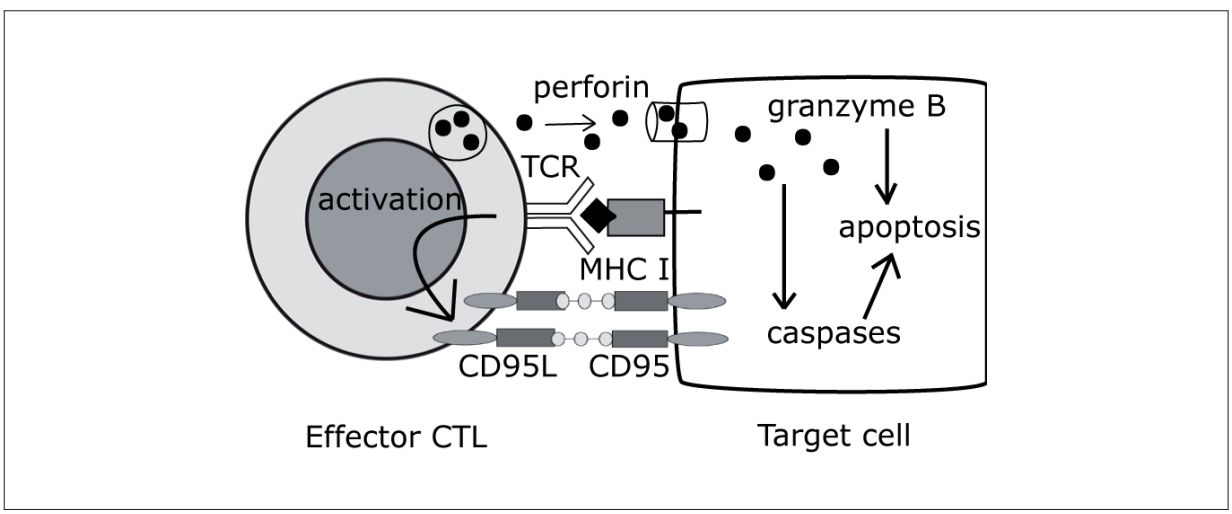

FIGURE 4. CTL CYTOTOXITY CAN BE MEDIATED BY TWO DISTINCT PATHWAYS

One mechanism is via secretion of perforin and granzyme B from cytolytic granules. Perforin creates pores in the membrane of the target cell to enable granzyme B entry into the cell. Granzyme activates caspases that induce apoptosis. The second mechanism is via interaction between CD95 (Fas) and CD95L (FasL). TCR-mediated activation induces CD95L expression on the CTL. Binding of CD95 on the target cells will induce sequential caspase activation, leading to apoptosis.

involved in control of cell proliferation and homeostasis among other cells.

Regulatory T cells

Regulatory T cells (Treg), include more than one cell type critical in the maintenance of peripheral TOLERANCE, down-modulation of the amplitude of an immune response, and prevention of AUTOIMMUNE DISEASES. There is enough evidence at present to conclude that Treg participate in all cell-mediated immune responses, directly affecting Th1, Th2, Th17, CTL, and B cell reactions against "self" and "foreign" Ag. The mechanisms by which Treg exert their function are still not completely clear, but immunosuppressive CYTOKINES such as TGF-β, IL-10 and IL-35 play an important role.

Although the majority of Treg appears within the $CD4^+$ T cell set, suppressor activity was also reported among $CD8^+$ T cells. Over the last few years, however, most attention was focused on $CD4^+$ regulatory cells and particularly the nTreg, which are characterized by constitutive expression of the α-chain of the IL-2 RECEPTOR (CD25) and the transcription factor Foxp3 [9]. nTreg arise from the thymus and represent about 10% of the total CD4 population.

Foxp3 is essential in the development and function of nTreg. The absence of functional Foxp3 results in severe systemic AUTOIMMUNE DISEASES in mice and man. Foxp3 inhibits IL-2 transcription and induces up-regulation of Treg-associated molecules, such as CD25, CTLA-4 and GITR [10], that can down-regulate the immune response of adjacent cells.

In addition to nTreg, iTreg develop in the periphery from naïve $CD4^+$ T cells in the presence of TGF-β and IL-10, or in the absence of COSTIMULATION, especially in mucosal tissues. Within the population of iTreg the heterogeneity is even more complex. Tr1 cells [11] depend on IL-10 for their induction and their suppressive action, whereas Th3 cells [12] depend on TGF-β for their suppressive action.

The inhibitory effect of all Treg primarily requires stimulation of the TCR. Upon activation, cells may mediate their function *via* direct cell contact through inhibitory molecules such as CTLA4, but they may also function *via* secretion of IL-10 and TGF-β. IL-10 can suppress differentiation of Th1 and Th2 cells directly by reducing IL-2, TNF-α and IL-5 production, and also indirectly by down-regulating MHC and COSTIMULATORY MOLECULES on APC, thereby reducing T cell activation. The mechanism of suppression will most likely depend on the type of Treg, the nature of the immune response, the Ag and the site of INFLAMMATION (Fig. 5) [13].

Mechanisms of T cell activation

Antigen presentation

Antigenic peptides are derived by different molecular mechanisms of Ag processing, from pathogens residing either in the cytosol or in vesicular compartments of the infected cell. MHC class I molecules bind to the antigenic peptides, which originate in the cytosol of APC as a result of a multimolecular complex of proteases (proteasomes) and are transported to the endoplasmic reticulum by TAP-1 and TAP-2 (transporter associated with Ag processing-1 and -2). The newly assembled MHC/peptide complexes in the endoplasmic reticulum are then translocated through the Golgi to the cell surface. Virtually all cells of the body express MHC class I molecules at different levels, and thus present antigenic peptides to $CD8^+$ CTL and become potential targets of destruction, depending on the Ag.

MHC class II molecules, in contrast, bind peptides deriving from pathogens that appear in intracellular vesicles of the cell or from extracellular proteins internalized by endocytosis. MHC class II molecules are transported from the Golgi to endosomes and lysosomes as a complex bound to the non-polymorphic invariant chain instead of a peptide. Subsequently, the invariant chain is degraded and replaced with peptides generated by vesicular acid proteases at acid pH in the endosomal compartments. MHC class II/peptide complexes appear on the surface of only a few types of immune cells, including MACROPHAGES, B cells, and DC [14].

Another important mechanism is cross-presentation of Ag, a process in which "professional" APC may present an Ag transferred from other cells. This

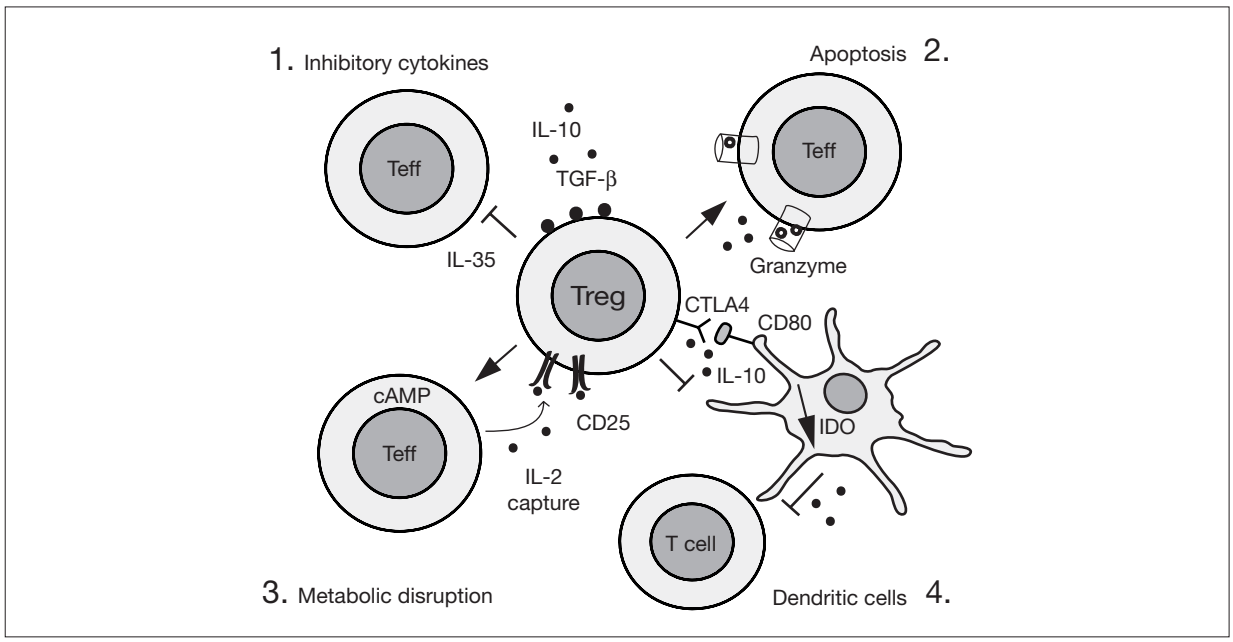

Figure 5. Several mechanisms mediate Treg cell function
1: Inhibitory cytokines such as IL-10 TGF-B and IL-35 can suppress T cell activation. 2: In some cases, cytolysis has been described as a potential suppressive mechanism, killing effector cells in a granzyme A- and B-dependent fashion. 3: Cytokine deprivation, through binding of IL-2, leads to metabolic disruption of target cells or direct cAMP mediated inhibition. 4: DC are targeted via direct cell-cell interactions, via CTLA4 (for example) or via suppressive cytokines such as IL-10.

enables extracellular Ag to be presented by MHC class I and to activate CTL. Several studies have shown that DC can actually initiate a T cell response against MHC class I-restricted Ag by cross-presentation. Cross-presentation also may serve as a mechanism for T cell TOLERANCE to self-Ag in the periphery [15].

Molecular mechanisms of T lymphocyte activation

Activation of naïve T cells is the most critical step in developing immunity and requires a complex interaction of TCR, CO-RECEPTORS, and accessory molecules on the surface of the T cell with corresponding ligands on the APC (Fig. 6). TCR-Ag/MHC interaction provides an Ag recognition step and initiates intracellular signaling. CO-RECEPTORS such as CD4 and CD8 assist the TCR signal. COSTIMULATORY MOLECULES such as CD28 and CTLA-4 initiate their own intracellular signals that enhance or modulate the TCR signal. Accessory molecules such as LFA-1 or CD2 provide adhesion at the cell contact site, strengthening the interaction between the T cell and APC and allowing sustained signal transductions. The αβ chains of TCR are non-covalently associated with invariant chains of the CD3 complex (ζ, δ, ε, and γ) (Fig. 2). Intracellular parts of CD3 chains include one or multiple immunoreceptor tyrosine-based activation motifs (ITAMs). ITAMs provide sites of interaction with protein tyrosine kinases (PTK) that propagate the signaling events [16].

Src family PTK *Fyn* and *Lck* phosphorylate ITAMs upon TCR cross-linking by Ag/MHC, and fully phosphorylated ITAMs recruit PTK ZAP-70 to the complex *via* their SH2 domains. This allows LCK to transpho-

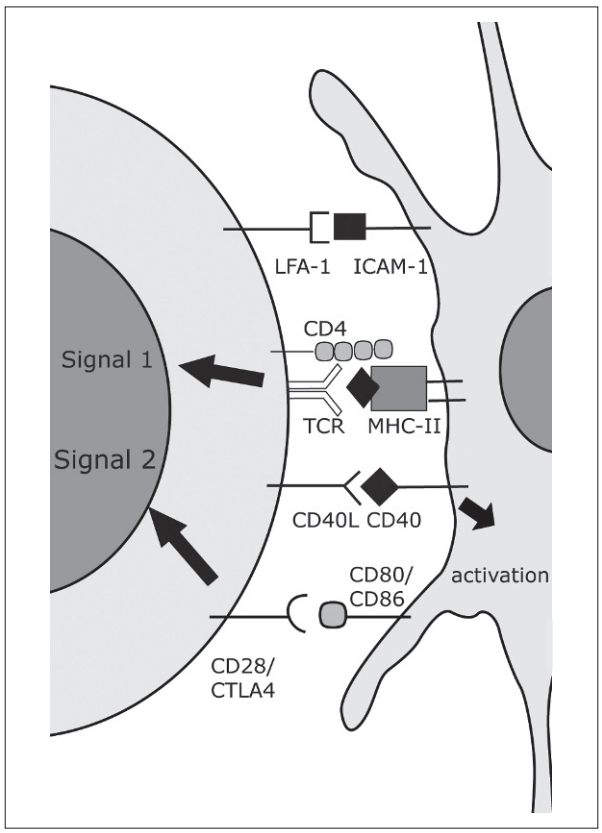

Figure 6. Effective T cell activation requires interaction with multiple surface receptors on both T cells and APC

Binding of MHC class II peptide complex to the TCR and CD4 induces signal 1 in the T cell. Positive costimulation (signal 2) is provided by binding of CD80 or CD86 to CD28, whereas binding to CTLA4 will inhibit T cell activation. Other interactions, such as binding of LFA-1 and ICAM-1, will ensure further intensified cell-cell interactions. Binding of CD40 and CD40L will induce an activating signal in the APC, enhancing the expression of MHC molecules and costimulatory receptors.

sphorylate and to activate ZAP-70. The activated ZAP-70 interacts and phosphorylates SLP-76, and LAT. SLP-76 appears to be involved in actin cytoskeleton changes, while LAT is a membrane-associated protein that upon phosphorylation provides binding sites for a number of critical signaling proteins, including Grb2, Ras, and PLC-γ. PLC-γ plays a critical role in regulation of Ca^{2+} flux as it cleaves 4,5-biphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) upon activation by PI3 kinase. DAG stimulates PKC, while accumulation of IP3 is the initial trigger for release of intracellular Ca^{2+} that, in turn, triggers the opening of the plasma membrane Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Cascade of the signaling actions eventually results in activation of transcription factors including NF-AT, ELK-1, Jun, and ATF-2 and immune GENE EXPRESSION.

Although the first phosphorylation events occur within a few seconds of TCR cross-linking, the sustained contact and interaction of T cells with APC is required for full T LYMPHOCYTE activation. Recent studies of TCR engagement have focused on immunological synapse (IS)-dynamic clustering of different surface molecules at the contact point between T cell and APC involving TCR/CD3, co-RECEPTORS, and accessory molecules [17]. The latest studies of IS reported a ring-type structure formed by TCR-Ag/MHC complexes around a cluster of LFA-1 and intercellular ADHESION MOLECULE-1 (ICAM-1) followed by inversion of this structure, relocation of TCR/pMHC to the center, and formation of spatially segregated regions of supramolecular activation complexes (SMAC) (Fig. 7). Mature IS contain central SMAC (c-SMAC), a cluster of TCR bound to Ag/MHC, and CD4 or CD8, CD3, CD2, CD2AP, CD28, PKCθ, and PTK*Lck*. c-SMAC is surrounded by peripheral SMAC, which contains LFA-1, ICAM-1, and talin. Thus, IS formed on the cell surface may provide prolonged cellular interaction and sustained signaling leading to the Ca^{2+} flux, actin cytoskeleton reorganization, and full-blown T cell activation. It was found that accumulation of cytolytic granules in CTL is directed toward IS and that release of the granules takes place within p-SMAC.

Tolerance

An essential part of T CELL-MEDIATED IMMUNITY is the development of non-responsiveness toward naturally occurring self-Ag, while mounting effective immune responses against "foreign" Ag [18]. Breakdown of self-TOLERANCE will result in the development of AUTOIMMUNE DISEASES. Self-reactive T cells, both $CD4^+$

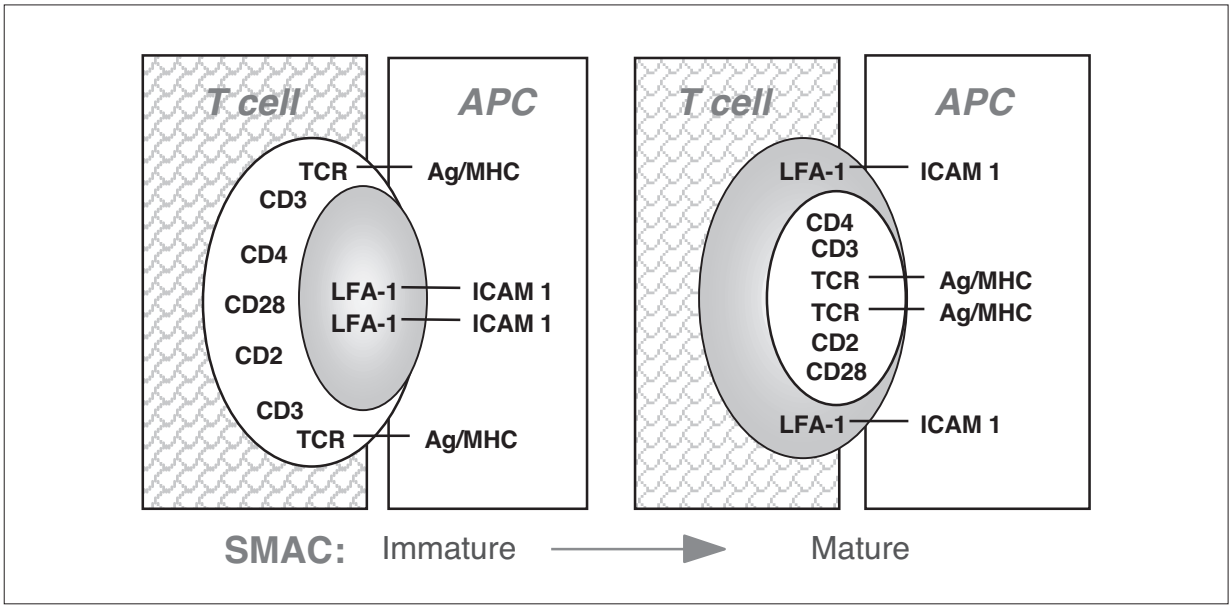

Figure 7. Receptor clusters of surface molecules

Schematic view of receptor clusters of different surface molecules forming a supramolecular activation complex (SMAC) on the membrane of the T cell at the site of interaction with the APC. The SMAC changes during the activation process, inverting central and peripheral composition.

and $CD8^+$, have been shown to be responsible for initiating and mediating tissue damage in many experimental animal models of organ-specific AUTOIMMUNITY as well as in human studies.

Immunological TOLERANCE is achieved by different mechanisms at different stages. Initially, potential self-reactive T LYMPHOCYTES are deleted during T cell development in the thymus. High-AFFINITY interaction of TCR on immature thymocytes with self-Ag on thymic stromal cells results in APOPTOSIS and elimination of such T cells in the process known as negative selection. T cells with TCR of low to moderate AFFINITY to self-Ag escape from the thymus and migrate to the periphery. These T cells are normally "ignorant" to self-Ag or develop TOLERANCE after initial activation.

Although the Ag-specific TCRs of T cells do not possess an intrinsic mechanism to distinguish self from non-self peptides, the activation by self-Ag is different to that by "foreign" Ag, mainly due to the absence of COSTIMULATORY SIGNALS from non-activated APC. This is in contrast to activated APC that up-regulate COSTIMULATORY MOLECULES during INFLAMMATION, infections, or other pathological conditions. Partial activation of T cells in the absence of COSTIMULATORY SIGNALS leads, instead of activation, to the state of T cell unresponsiveness toward further stimulation, also known as anergy [19].

In most cases, COSTIMULATORY MOLECULES will direct T cell response towards either activation or TOLERANCE. Simple absence of COSTIMULATORY SIGNALS was shown to induce anergy in effector T cells *in vivo* and *in vitro*, while naïve T cells may require a negative signal of CTLA-4 engagement to develop anergy and become tolerant.

Self-reactive cycling T cells may also undergo programmed cell death after re-exposure to the same Ag in a process called activation-induced cell death (AICD). AICD is mediated by death RECEPTORS (FAS/FAS-LIGAND interaction of $CD4^+$ T cells and by TNFRII/TNF interaction of $CD8^+$ T cells) that involve interaction of caspase-dependent, death-inducing signaling complexes (DISC).

Peripheral TOLERANCE can also be controlled by immune cytokine divergence and by Treg cells. Both NATURAL and adaptive $CD4^+$ regulatory cells have been implicated in the regulation of the autoimmune response. Thymus-derived $CD25^+$ nTreg cells suppress other types of cell activation by largely unknown mechanisms. They require strong COSTIMULATORY SIGNALS for induction and maintenance, with Foxp3 expression. Adaptive (Ag-induced) Treg cells are generated in the periphery by sub-optimal antigenic signals and rely on CYTOKINES such as IL-10 and TGF-β for suppression. These cells of varying phenotype often appear under special conditions such as chronic viral infections. Treg present new possibilities for the treatment of autoimmune disorders and for the maintenance of transplanted organs.

Summary

T CELL-MEDIATED IMMUNITY includes priming of naïve T cells, effector functions of activated T helper cells and CTL, and long-term persistence of memory T cells. Development of an effective immune response requires proper activation of T LYMPHOCYTES by APC in secondary LYMPHOID ORGANS and migration of the responding T cells to the sites of Ag presence in the body. The efficiency of T cell activation in LYMPHOID ORGANS depends on the concentration of an antigenic peptide and AFFINITY of TCR toward the Ag/MHC complex, and is facilitated by inflammatory stimuli, COSTIMULATORY SIGNALS, and CYTOKINES. $CD8^+$ naïve T cells develop into effector CTL after interaction with APC, while $CD4^+$ naïve T cells differentiate into T helper cells of major T helper types: Th1 (producing IL-2, IFN-γ, TNF-α, and LT-α) or Th2 (IL-4, IL-5, IL-6, IL-10, and IL-13).

Absence of inflammatory stimuli may induce insufficient activation of DC, resulting in induction of anergy and APOPTOSIS among T cells instead of activation and productive response. This may serve as a mechanism of TOLERANCE to self-Ag. Circulation and extravasation of T LYMPHOCYTES are orchestrated by multiple adhesion RECEPTORS whose expression and avidity is modulated by CYTOKINES and CHEMOKINES. In the process of mediating effector functions, some activated T cells undergo activation-induced cell death (AICD), while others undergo activated T cell autonomous death after the INFLAMMATION wanes, thus terminating the immune response. Only a small population of Ag-specific memory cells remains in LYMPHOID ORGANS and throughout the tissues for a prolonged period after the immune response is over. When exposed to the Ag a second time, memory cells rapidly acquire and mediate effector functions, thereby preventing spread of pathogenic infection.

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A3

Antibody diversity and B lymphocyte-mediated immunity

Ger T. Rijkers

Towards the end of the 19th century, Koch and Ehrlich discovered that the serum of immunized animals contained substances (antitoxins) with the ability to neutralize the toxins of diphtheria and tetanus. At Christmas 1891 a group of children received diphtheria antitoxin, which cured them from this otherwise fatal disease. These experiments demonstrated that immunization can induce the formation of humoral substances, which have the ability to protect against infectious diseases. Half a century later, in 1952, Bruton described a patient with severe and recurrent respiratory tract infections and an AGAMMAGLOBULINEMIA. This milestone demonstrated the significant role of IMMUNOGLOBULINS in the defense against infections. Later on, through the pioneering work of Max Cooper and others, it was shown that B LYMPHOCYTES are the cells that produce ANTIBODIES, and that patients such as the one described above (X-linked AGAMMAGLOBULINEMIA or XLA) fail to produce ANTIBODIES because they lack B LYMPHOCYTES; B lymphocyte development in the BONE MARROW stops at the pre-B cell stage. Forty years after the initial discovery, the molecular basis for this disease was found: XLA is caused by structural defects in the gene encoding an enzyme that has been termed Bruton's tyrosine kinase (Btk).

Antibodies and immunoglobulins

Host defense against infections with microorganisms depends on the complex interplay between cells and proteins of the IMMUNE SYSTEM, which together are capable of recognizing and specifically interacting with molecular structures of the microorganism. The proteins involved in this process include TOLL-LIKE RECEPTORS, DEFENSINS, collectins (such as mannose-binding LECTIN), surfactant proteins, and IMMUNOGLOBULINS. Among these proteins, the IMMUNOGLOBULINS are special because of their extreme diversity in primary structure. It is estimated that up to 10^{12} different immunoglobulin molecules can be formed (see also below). This endows the host with a large spectrum of defense molecules that can bind specifically to virtually any given microorganism. The IMMUNOGLOBULINS in serum comprise approximately 10–20% of total serum proteins; upon electrophoresis they end up in the gamma region, hence the alternative name of gammaglobulins for IMMUNOGLOBULINS.

ANTIBODY molecules are IMMUNOGLOBULINS and IMMUNOGLOBULINS are ANTIBODIES. Still, it can be confusing to intermix these terms. The term ANTIBODY should only be used for IMMUNOGLOBULINS with known SPECIFICITY for antigen, such as anti-bloodgroup A ANTIBODIES or anti-measles ANTIBODIES. Furthermore, the term "ANTIBODY" is also used when describing the interaction of an immunoglobulin with antigen. The term "immunoglobulin" is used when dealing with molecular or biochemical characteristics of these proteins.

Structure of immunoglobulins

IMMUNOGLOBULINS are glycoproteins with a basic structure of four polypeptide chains, comprising two identical heavy chains of 400–500 amino acids and two identical light chains of ca. 200 amino acids (Fig. 1). These chains are held together by disulfide bridges and non-covalent protein-protein interactions. On the basis of the primary structure of the heavy chains, the IMMUNOGLOBULINS are divided into five classes or ISOTYPES, namely IgG, IgA, IgM, IgD, and IgE. IgG is further subdivided into four subclasses IgG1, IgG2, IgG3, and IgG4, while IgA is subdivided into

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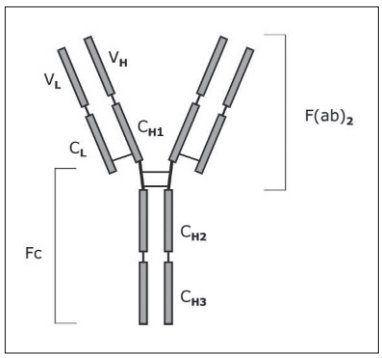

Figure 1. Schematic structure of an IgG molecule
The immunoglobulin molecule has 2 identical heavy chains, each composed of 3 constant domains (C_{H1}, C_{H2}, C_{H3}), and a variable domain (V_H). Two identical light chains [composed of a constant (C_L) and a variable domain (V_L)] are connected to the heavy chains by disulphide bonds. Fragments of the molecule that can be obtained after treatment with proteolytic enzymes are indicated as F(ab)$_2$, the antigen-binding fragment, and Fc, the fragment that can be obtained in crystallisable form.

two subclasses IgA1 and IgA2. There are two types of light chains, kappa (κ) and lambda (λ) chains. Heavy chains and light chains each have a domain structure. Heavy chain molecules consist of four domains, three of them are termed constant domains (C_{H1}, C_{H2}, C_{H3}; see Fig. 1) because of the minor variation in amino acid sequence, while the fourth domain shows considerable sequence variation (V_H). Light chains have a two domain structure (C_L and V_L). The combination of the variable domains of one heavy and one light chain determines the specific recognition of a microorganism and the binding to it. The constant domains determine the biological activity of the formed ANTIBODY-microorganism complex (see below: Biological functions of ANTIBODIES). In contrast to IgG, the heavy chains of IgM and IgE consist of four constant domains and one variable domain. IgG as shown in Figure 1 is a monomeric structure. IMMUNOGLOBULINS can also appear as multimeric structures. IgM consists of five monomers (pentamer) and IgA appears in serum predominantly as a monomer but can also appear as a dimer. In secretion fluids and on mucosal surfaces of the respiratory and gastrointestinal tract, IgA is present as secretory IgA. Secretory IgA is dimeric IgA coupled to a J chain and a secretory component that is important for the transport of IgA through the epithelial cells to external secretions and the protection of the secreted molecule from proteolytic digestion.

Generation of antibody diversity

The combination of the variable domains of the heavy and the light chain forms the binding site for the antigen [1]. IMMUNOGLOBULINS are capable of recognizing a wide variety of different ANTIGENS because of the large variation in amino acid sequences within the variable domains. The genes encoding the V_H domain are organized in clusters each of which encodes parts of the variable domain (gene segments). In precursor B LYMPHOCYTES, during the process of gene rearrangement, three gene segments are joined together, one so-called variable (V) segment, one diversity (D) segment and one joining (J) segment. In humans, there are more than 60 V gene segments, about 30 D and 6 J gene segments. The three segments are joined together in random combination, allowing already more than 10 000 different combinations at this level (Fig. 2a, page 32). This process requires the activity of two enzymes, the recombinase activating genes RAG-1 and RAG-2. The rearrangements take place in a defined order; first a given D gene segment joins with a given J gene segment and subsequently a given V gene segment is joined to the combined DJ sequence. In addition, nucleotides at the ends of the gene segments can be removed enzymatically by nucleotidases (NT), while extra nucleotides can be inserted by the enzyme terminal-deoxynucleotidyl transferase (TdT). These processes of deletion or addition of nucleotides change the germline nucleotide sequences and thus contribute to greater diversity. Next, the gene segments encoding the variable domain and the segments encoding the constant part are joined and transcribed into mRNA, spliced together and translated into a complete heavy chain. During this joining process several nucleotides can also be added or deleted. A similar process occurs for the immuno-

globulin light chain, except that the variable region of the light chain is composed of V and J segments only. The amino acid sequence variability in the V regions is especially pronounced in three hypervariable regions; these are the places where the V, D, and J segments are joined. Localized areas of these hypervariable regions of the H and L chains interact to form antigen-binding sites (i.e., the COMPLEMENTARITY DETERMINING REGIONS, CDR1, CDR2 and CDR3).

Early during B lymphocyte development, rearrangements of the coding segments of the variable regions take place. Daughter cells derived from such a B lymphocyte form a CLONE of B LYMPHOCYTES, which all express identical IMMUNOGLOBULINS expressing this particular combination, and therefore should all have identical antigen-binding capacity. However, additional diversity can be generated by SOMATIC MUTATIONS and by altering the heavy chain constant region, called CLASS SWITCHING (illustrated in Fig. 2b, page 32). CLASS SWITCHING is regulated by two enzymes: activation-induced cytidine deaminase (AID) and uracil-*N*-glycosylase (UNG). The first transcript to be produced as a B lymphocyte develops, after VDJ joining, contains the exon for V_H and the exon for C_H of the μ chain, resulting in expression of IgM and production of IgM when stimulated by binding of an antigen. When the B lymphocyte is further stimulated by an antigen, the class of ANTIBODY being produced changes. DNA encoding a different constant domain gene segment is joined to the original V_H exon, while intervening DNA encoding the μ heavy chain gene segment is eliminated and forms the so-called excision circle (see also Fig. 2b). Thus, the B lymphocyte will produce another immunoglobulin molecule with identical SPECIFICITY for the antigen [2]. At the same time, but also independent of the process of CLASS SWITCHING, mutations in the variable region may arise. This results in B LYMPHOCYTES with (small) differences in the AFFINITY of the ANTIBODY molecule that is expressed on the membrane, either lower or higher AFFINITY. When antigen concentration becomes low, only the B LYMPHOCYTES with high AFFINITY for that particular antigen will be activated. This increase in the AFFINITY of ANTIBODIES during a humoral immune response is called AFFINITY MATURATION.

In the absence of specific antigen, mature B LYMPHOCYTES survive in the peripheral circulation for only a few days. Cells that do not encounter antigen within this period of time undergo APOPTOSIS (see Box 1). This is necessary to maintain an optimal and more or less constant number of B LYMPHOCYTES in the peripheral circulation. However, when immature B LYMPHOCYTES develop in an environment containing a "self"-antigen, the B LYMPHOCYTES will also undergo APOPTOSIS and thus be deleted from the REPERTOIRE.

B cell receptor and signal transduction

B lymphocyte activation is initiated by specific recognition of antigen by the antigen RECEPTOR, i.e.,

Box 1. Apoptosis

There are two different ways in which cells can die. Cells can die in an uncontrolled manner, termed necrosis, because they are damaged by injurious agents such as toxic agents resulting in cell lysis. Alternatively, they can commit suicide in an active, gene-regulated process. Cells that undergo apoptosis, also called programmed cell death, undergo a characteristic series of changes. The cells shrink, form bubble-like blebs on their surface, and the chromatin (DNA + proteins) in the nucleus is degraded forming a vacuolar nucleus. Cells undergo apoptosis for different reasons. One entails normal development, such as formation of fingers and toes of a fetus, through apoptosis of the tissue between them. Another is for the benefit of the organism. Cells with DNA damage, tumor cells, or cells of the immune system that are able to attack their own body tissues need to be cleared from the organism by apoptosis. Defects in the apoptotic machinery are associated with autoimmune diseases such as rheumatoid arthritis and lupus erythematosus.

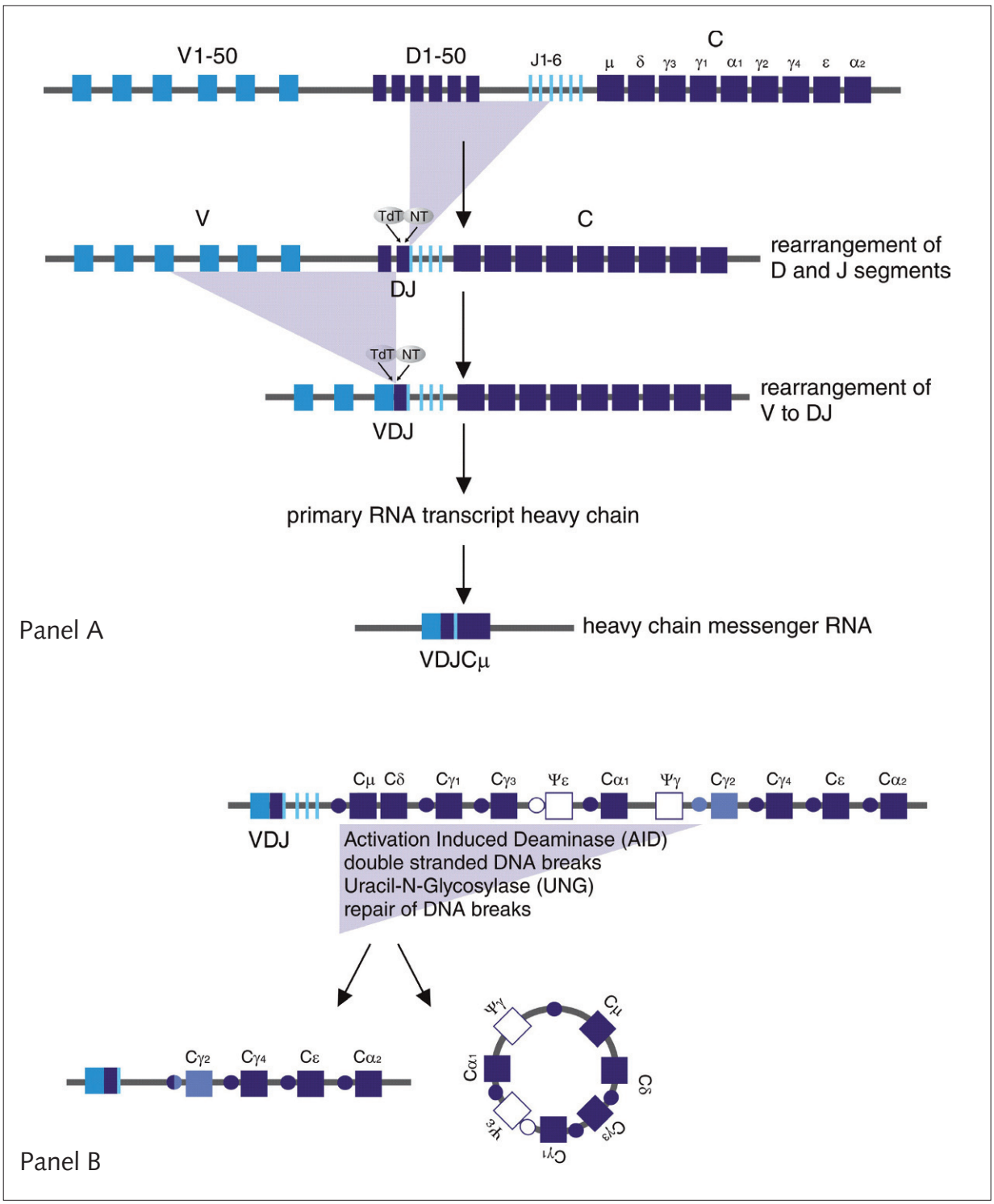
V1-50
D1-50
J1-6
C
μ δ γ3 γ1 α1 γ2 γ4 ε α2
V
TdT NT
C
DJ
rearrangement of
D and J segments
TdT NT
VDJ
rearrangement of
V to DJ
primary RNA transcript heavy chain
VDJCμ
heavy chain messenger RNA
Panel A
Cμ Cδ Cγ1 Cγ3 Ψε Cα1 Ψγ Cγ2 Cγ4 Cε Cα2
VDJ
Activation Induced Deaminase (AID)
double stranded DNA breaks
Uracil-N-Glycosylase (UNG)
repair of DNA breaks
Cγ2 Cγ4 Cε Cα2
Ψγ1
Cμ
Cδ
Cγ3
Cγ1
Ψε
Cα1
Panel B

Figure 2. Molecular mechanisms of rearrangement of the immunoglobulin gene segments (A) and class switch recombination (B)

(A) During early B lymphocyte development, rearrangement of one of the D gene segments to one of the J segments takes place under regulation by the recombinase activating genes RAG-1 and RAG-2. Subsequently, rearrangement of one of the V gene segments to the DJ segment occurs. Before D is joined to J and DJ to V, the enzymes, terminal deoxynucleotidyl transferase (TdT) and endonucleotidase (NT), can add (TdT) or remove (NT) extra nucleotides to or from the open ends of the DNA strands. The primary RNA transcript is processed by splicing the VDJ segment to a constant region gene segment (C), in this example Cμ.

(B) Every C_H gene segment is preceded by a so-called switch region (indicated by bullets in the figure). During class switch recombination, double-stranded DNA breaks occur in the switch regions of Cμ and the C_H gene segment of the isotype to which the switch occurs (γ_2 in this example). The enzymes, activation-induced deaminase and uracil-N-glycosylase, play a (yet-undefined) role in this process.

membrane-bound immunoglobulin (mIg). Resting, primary B LYMPHOCYTES express two ISOTYPES of mIg: mIgM and mIgD. Both mIgM and mIgD (as well as other mIg ISOTYPES; see below) are expressed on the cell surface in association with Igα and Igβ molecules; collectively such a complex is called the B cell RECEPTOR (BCR) complex. Igα (CD79a) and Igβ(CD79b) are the protein products of the MB-1 and B29 genes, respectively, and both belong to the Ig superfamily [3]. Igα and Igβ fulfill at least three different functions: they are required for expression of mIg on the surface of the B lymphocyte, they act as transducer elements coupling the antigen RECEPTOR to intracellular signaling molecules by virtue of the immunoRECEPTOR tyrosine-based activation motif (ITAM) (see below), and they contain sequences for efficient internalization of antigen.

One of the first signs of cellular activation after antigen-induced ligation of the BCR is the increase in the activity of protein tyrosine kinases (PTKs; see Box 2). Because the cytoplasmic domains of mIgM and mIgD consist of only three amino acids, it could be assumed that Igα (cytoplasmic domain of 61 amino acids) and Igβ (48 amino acids) serve a role in signal transduction. Of crucial importance for signal transduction is the ITAM, present in the cytoplasmic domain of Igα and Igβ (Fig. 3). This amino acid motif resides in a 26-amino acid sequence and consists of a tyrosine (Y) followed, two residues later, by a leucine (L) or isoleucine (I), a sub-motif that is repeated once after every six to seven variable residues. The complete ITAM also contains two aspartate (D) or glutamate (E) residues at characteristic positions (Fig. 3). The ITAM is found in Igα and Igβ, in the CD3γ and CD3δ chains of the T CELL RECEPTOR (TCR) complex (see chapter A2), and in the γ chain of the Fcε RECEPTOR type I. ζ chains of the TCR contain three copies of the ITAM. More or less

Box 2. Protein tyrosine kinases (PTK)

PTK is an enzyme that catalyzes the phosphorylation of tyrosine residues in proteins using adenosine triphosphate (ATP) or other nucleotides as phosphate donors. PTK activity is defined as catalysis of the reaction: ATP + protein tyrosine = adenosine diphosphate (ADP) + protein tyrosine phosphate. Cell-to-cell signals for growth, differentiation, adhesion, motility, and death are frequently transmitted through tyrosine kinases. PTKs represent a diverse and rapidly expanding superfamily of proteins, including both transmembrane receptor tyrosine kinases and soluble cytoplasmic enzymes. Activation of the PTK domain of either class of PTK enzymes results in interaction of the protein with other signal-transducing molecules and propagation of the signal along a specific signal transduction pathway.

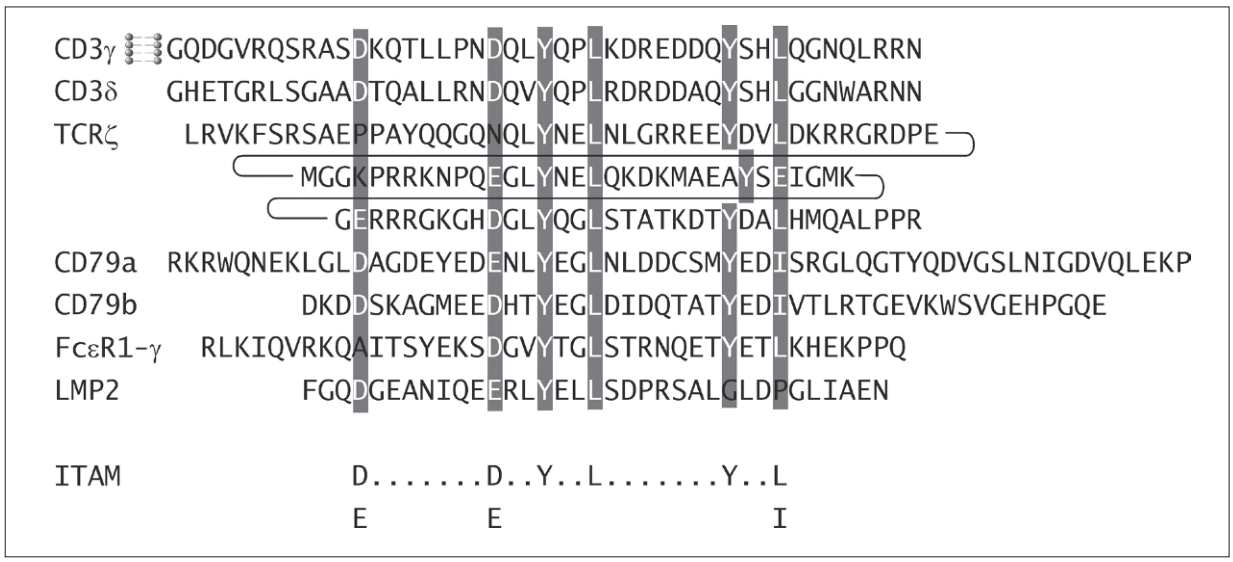

Figure 3. The immunoreceptor tyrosine-based activation motif (ITAM)
The amino acid sequence (given in the single letter code) of the cytoplasmic domains of human CD3γ, CD3δ, the TCRζ chain, Igα (CD79a), Igβ (CD79b), the γ chain of the type I Fcε receptor (FcεRI-γ) and of the EBV encoded LMP2 protein. Note that the cytoplasmic domain of TCRζ is depicted in three interconnected parts in order to allow the alignment of the three copies of the ITAM within the sequence. See text for further explanation.

truncated forms of ITAM are present in CD22 and Fcγ RECEPTOR type II. In addition, a virus-encoded protein, LMP2 from Epstein-Barr virus, contains an ITAM. The central role of ITAM in cellular signaling through the BCR complex (as well as through the TCR complex) has become apparent from studies in which mutants with a single amino acid-substituted RECEPTOR and CHIMERIC RECEPTOR molecules have been used.

Upon triggering of mIg, a number of cytoplasmic PTKs become associated with the BCR. These include kinases of the src family, such as lyn, fyn and blk, as well as the syk tyrosine kinase and Bruton's tyrosine kinase (Btk). The binding is mediated by the interaction of the src-homology 2 (SH2) domain within the tyrosine kinase with phosphorylated tyrosine residues within the ITAMs of Igα and Igβ. Note that this model suffers from a "chicken and egg" problem: if binding of SH2 domains occurs at phosphorylated ITAM tyrosines, how do ITAM tyrosines become phosphorylated initially? It has been found, however, that an alternative interaction is possible, not dependent on phosphotyrosine: the ten N-terminal residues of src kinases can interact with a specific sequence within the ITAM of Igα (DCSM).

Following phosphorylation of Igα and Igβ by the src family PTKs, syk is recruited and activated [4]. Binding of src and syk kinases to (phosphorylated) ITAMs triggers a series of downstream signaling events, in which adaptor proteins are involved. B cell adaptor molecules, such as B cell linker (BLNK) and Bam32, function as conduits to effectively channel upstream signals to specific downstream branches. These include activation of phospholipase Cγ2, of GTPase-activating protein, of MAP kinase (all through the N-terminal regions of lyn, fyn and blk), of phospholipase Cγl (through syk), of the guanine nucleotide-releasing factor Vav, and of p85 phosphoinositide-3-kinase (PI-3 kinase, through the SH3 domains of fyn and lyn). Activated PI-3 kinase in turn results in the phosphatidylinositol (3,4,5) triphosphate (PIP3)-mediated recruitment of Btk to the plasma membrane where it is involved in activation of phospholipase Cγ.

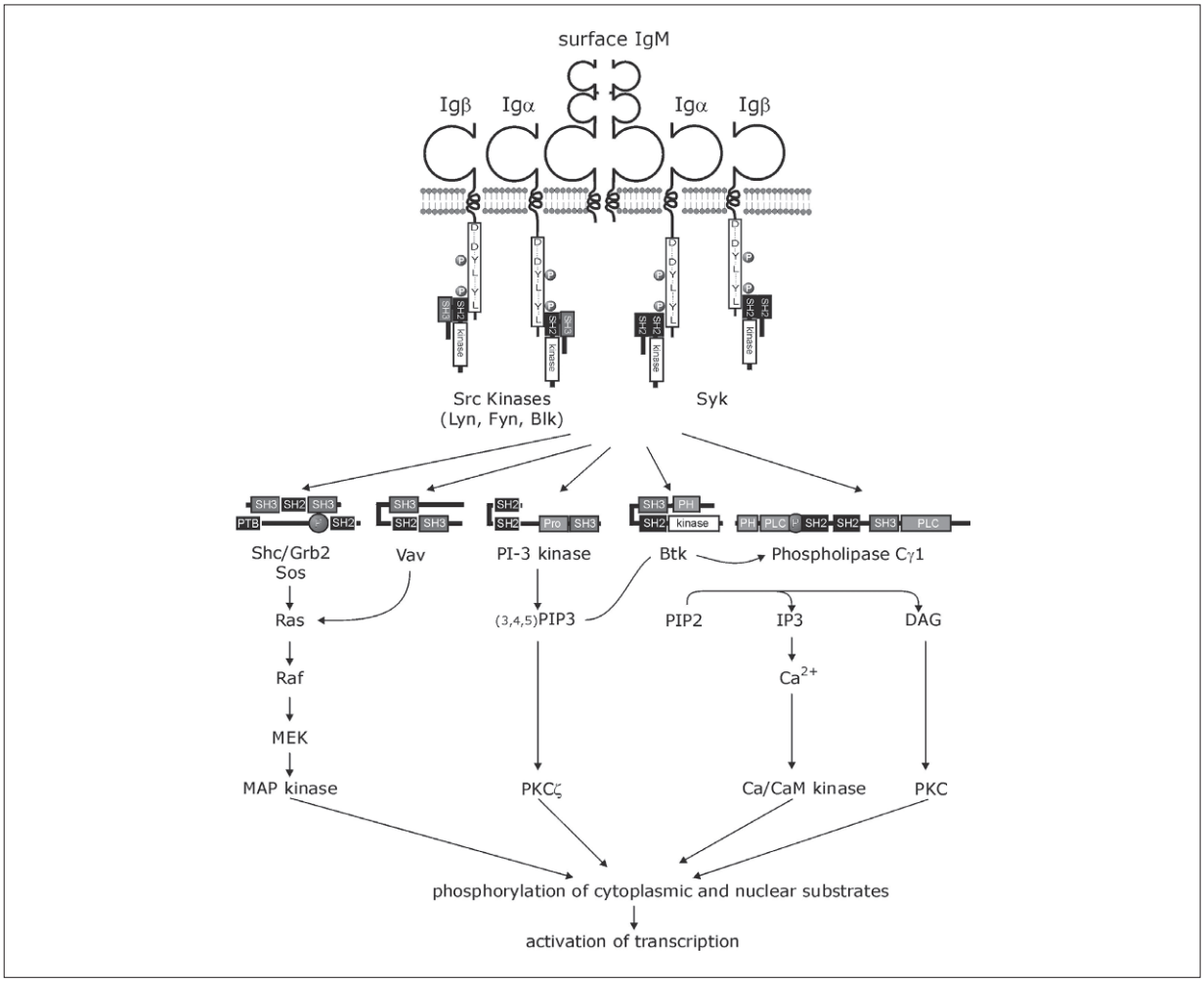

FIGURE 4. SIGNALLING THROUGH THE BCR COMPLEX

The membrane immunoglobulin is composed of disulfide-linked heavy and light chain molecules (only partially shown in the figure) flanked by non-covalently associated dimers of Igα and Igβ. Ovals in membrane immunoglobulin, Igα and Igβ, indicate homologous domains of immunoglobulin superfamily members. The cytoplasmic domains of Igα and Igβ contain the ITAM. Phosphorylation (P) of the tyrosine residues (Y) in the ITAMs allows src kinases (lyn, fyn, blk) and syk to associate with the BCR. Activated kinases lead to further phosphorylation of ITAMs, autophosphorylation as well as phosphorylation of a number of cell-signaling molecules. The latter include Shc and Grb2 (initiating the MAP kinase-signaling pathway), Vav (activating Ras and thus also leading to activation of MAP kinase), phospholipase Cγ1 [catalyzing the hydrolysis of phosphatidyl inositol diphosphate (PIP2)], PI-3 kinase [leading to generation of phosphatidyl inositol trisphosphate (PIP3)] and Btk. PIP2 hydrolysis generates inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 causes the release of Ca^{2+} from intracellular stores and the subsequent activation of Ca^{2+}/calmodulin-dependent protein kinase (Ca/CaM kinase). DAG, in the presence of phosphatidyl serine and high Ca^{2+}, activates protein kinase C (PKC). PIP3 activates the ζ isoform of PKC. All kinases described above phosphorylate cytoplasmic and nuclear substrates (including transcription factors), leading to activation of transcription and thus B lymphocyte activation.

Thus, originating from the BCR, several cytosolic PTKs are activated, resulting in the initiation of several distinct cell-signaling pathways (Ras, phospholipase C, PI-3 kinase) *via* various adaptor molecules (e.g., BLNK and Bam32). These signaling pathways result in the activation of a set of protein kinases, which in turn phosphorylate cytoplasmic and nuclear substrates, and ultimately activate transcription (Fig. 4).

B lymphocyte costimulation

Whereas the events described above are causally linked to B lymphocyte proliferation and differentiation into ANTIBODY-secreting plasma cells, in only a few cases is triggering of the BCR by specific antigen sufficient to ensure subsequent B lymphocyte activation and differentiation. In all other instances, involving the vast majority of naturally occurring ANTIGENS, the process of B lymphocyte activation and differentiation depends on activation of additional RECEPTORS on the B lymphocyte. A number of these RECEPTORS interact with counter-RECEPTORS on T LYMPHOCYTES, thus providing the structural basis for the interaction between these two cell types in the process of ANTIBODY formation. Other RECEPTORS on B LYMPHOCYTES have ligands that are also expressed on other cell types (such as MONOCYTES, endothelial cells etc.) or have soluble ligands.

A major co-RECEPTOR on B LYMPHOCYTES is CD40 [5]. This 50-kDa glycoprotein is a member of the so-called TNF RECEPTOR superfamily and is expressed on all mature B LYMPHOCYTES. The counter RECEPTOR for CD40 is the CD40 LIGAND (CD40L), a 39-kDa cell surface glycoprotein, which is expressed on activated T helper cells. The role of CD40 and CD40L for the process of B lymphocyte activation is depicted schematically in Figure 5. Antigen that is bound to the BCR is internalized, processed, and peptide fragments derived from the antigen are subsequently presented in combination with MAJOR HISTOCOMPATIBILITY COMPLEX (MHC; see Box 3) class II molecules expressed on the B lymphocyte surface. These peptides can be recognized by specific T LYMPHOCYTES, leading to T LYMPHOCYTE activation and thereby expression of CD40L. The interaction of CD40L with

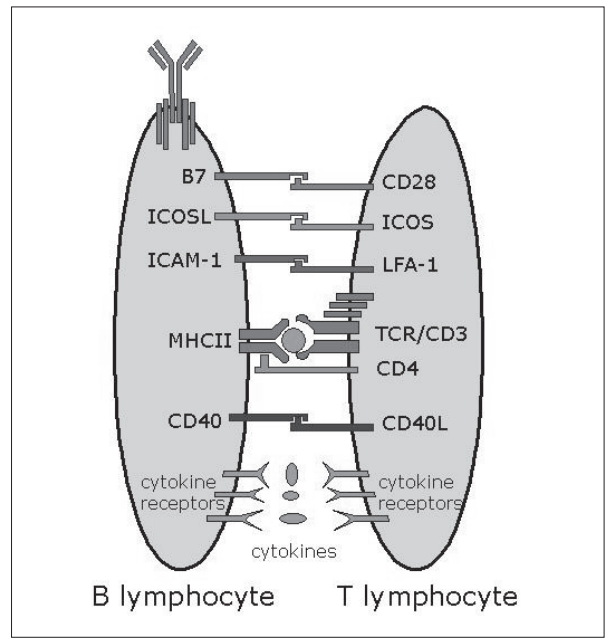

FIGURE 5. INTERACTION BETWEEN B AND T LYMPHOCYTE
A peptide/MHC class II complex expressed on a B lymphocyte can be specifically recognized by a T cell receptor expressed on a CD4+ T cell (signal 1). To induce an effective and sustained immune response, an additional signal is needed. CD40L interacts with the CD40 molecule on the B cell, resulting in an increased expression of B7. Ligation of CD28 by B7 provides a costimulatory signal (signal 2) needed for T cell activation and production of IL-2. Expression of ICOS is induced and the molecule interacts with its ligand. Subsequently, several types of cytokines can be produced and antibody production by B cells is stimulated.

CD40 results in progression of the B cell activation process, including acquisition of the capacity to proliferate in response to soluble CYTOKINES produced by the activated T LYMPHOCYTE. The signal received through CD40 is also important for the process of CLASS SWITCHING, the mechanism through which ANTIBODIES of immunoglobulin classes other than IgM are produced. The biological significance of the interaction between CD40 and CD40L is illustrated in a human immunodeficiency disease, the so-called X-linked hyper-IgM syndrome. Affected

Box 3. Major histocompatibilty complex (MHC)

MHC molecules, also known as human leukocyte antigens or HLA, are the products of a cluster of genes in the human DNA. There are two types of MHC molecules, MHC class I and MHC class II. MHC class I molecules designated as HLA-A, -B, and -C are expressed on all nucleated cells, while MHC class II molecules (HLA-DR, -DQ, and -DP) are primarily expressed on antigen-presenting cells (APC) like macrophages, dendritic cells and B lymphocytes. MHC molecules bind small protein fragments, called peptides, and form MHC/peptide complexes at the cell surface. Recognition of these MHC/peptide complexes by the T cell receptor is required for T cell activation. B lymphocytes, however, are also able to directly recognize antigens via their B cell receptor.

patients carry mutations in the CD40L gene, which result in the inability to produce antibodies other than IgM because the switch to IgG or IgA is not possible. This disease is thus an example of a defect in T lymphocytes that is reflected in impaired function of B lymphocytes.

Additional costimulatory receptor-counter receptor pairs contribute to successful interaction between B lymphocytes and T lymphocytes, such as CD28 and the inducible costimulator (ICOS) on T lymphocytes and B7.1/B7.2 (CD80/CD86) and ICOS ligand (B7h) on B lymphocytes, respectively [6]. As a result of CD40-CD40L interaction, the expression of B7 on the B lymphocyte is up-regulated. Ligation of CD28 by B7 provides a costimulatory signal that is required for T cell activation, proliferation, production of IL-2, and cell survival. Both MHC/peptide complex-T cell receptor interaction (signal 1) and CD28-ligand interaction (signal 2) are needed for the induction of a sustained immune response.

Cytokine regulation and isotype switching

The full stimulatory effect of the interaction between T lymphocytes and B lymphocytes depends not only on binding of cell surface receptors and counter receptors, but also on production of T cell cytokines that promote (various stages) of B lymphocyte proliferation and terminal differentiation into plasma cells. The soluble cytokines should not be considered merely as endocrine hormones, because they are secreted at the sites of direct cell-cell contact; therefore, the particular B lymphocyte engaged in cellular interaction with the relevant T lymphocyte benefits most from these growth and differentiation factors.

The cytokines that regulate B lymphocyte growth and differentiation predominantly include interleukin (IL)-4, IL-6, IL-10, and IL-21 [7, 8] (see also chapter A5). IL-4 acts as a costimulator for signals received through the BCR and CD40 in promoting B lymphocyte growth. IL-6 and IL-21 primarily regulate the differentiation of an activated B lymphocyte into an antibody-secreting cell. IFN-γ and IL-21 promote switching to IgA, while IL-10 and TGF-β drive activated B lymphocytes to IgA. Both IL-4 and the related cytokine IL-13 can cause switching to IgE and IgG4 production (Fig. 6).

The molecular mechanisms that govern the changes in the isotype of the immunoglobulin heavy chain used in the B lymphocyte have been described above. cytokines play a role in this process by making the DNA in switch regions accessible for the enzymes AID and UNG. The different switch regions are activated more or less cytokine-specifically: IL-21 promotes switching to IgG1 and IgG3, TGF-β and IL-10 to IgA1. IFN-γ regulates switching to IgG1, IL-4 and IL-13 to IgE. Two other cytokines (BAFF and APRIL) produced by macrophages, dendritic cells, and epithelial cells, drive T lymphocyte-independent isotype switching of B lymphocytes [9]. BAFF (B cell-activating factor) can bind to the BAFF receptor and to TACI (transmembrane activator and calcium-modulating cyclophilin ligand interactor) on B lymphocytes or to BCMA (B cell maturation antigen) on plasma cells (Fig. 7). APRIL (A proliferation-inducing ligand) binds to both TACI and BCMA.

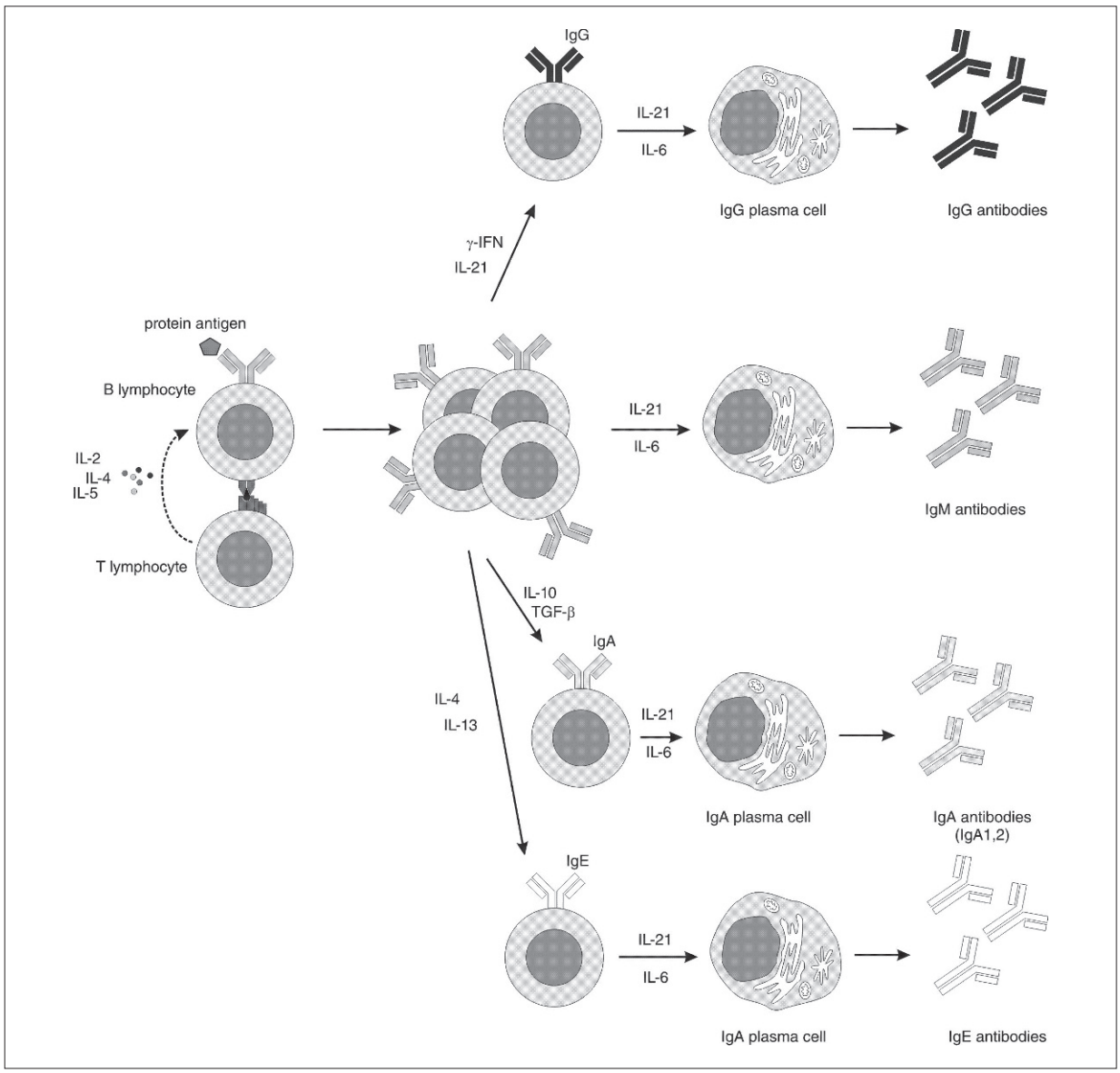

Figure 6. Cytokine regulation of B lymphocyte activation and class switching

T cell-independent B lymphocyte activation

In the above section the important role of interaction with T lymphocytes for the process of B lymphocyte activation has been emphasized. There is, however, a category of antigens that is unable to activate T lymphocytes, whereas B lymphocyte responses and induction of antibodies can be readily demonstrated. These types of antigens are called T cell-independent antigens, and major representatives are capsular poly-

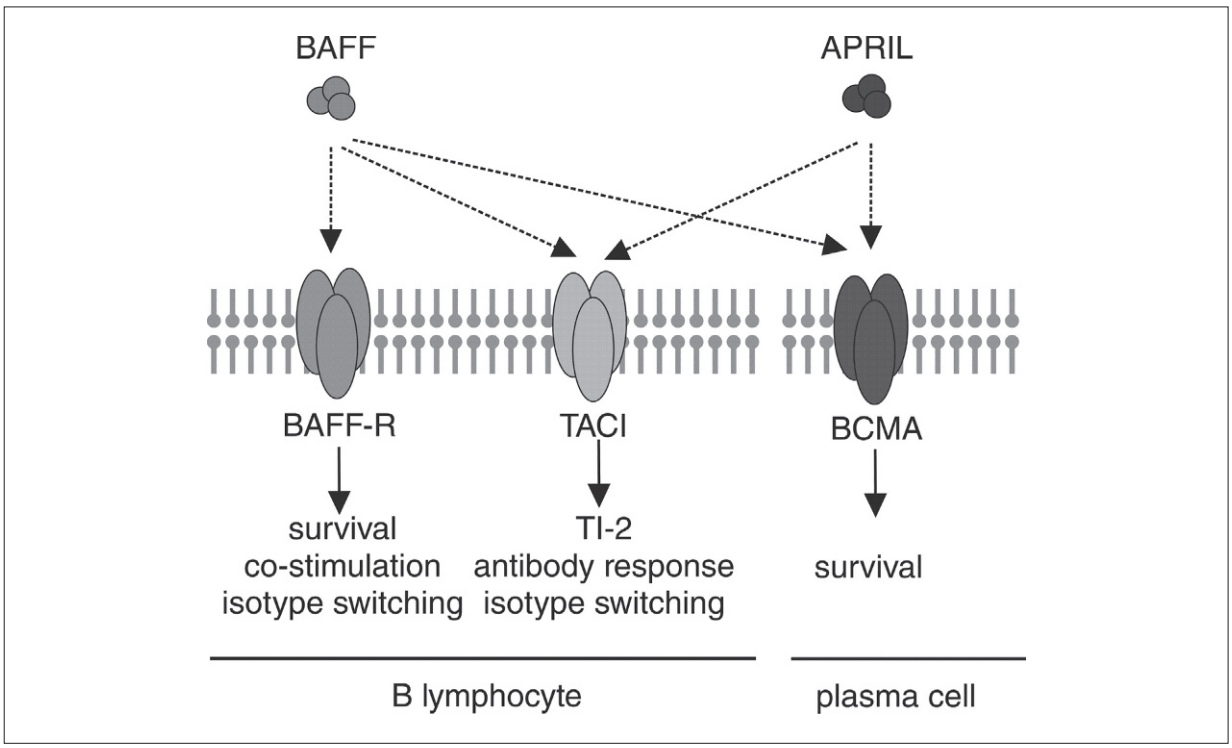

Figure 7. Regulation of B lymphocyte activation and function by BAFF and APRIL
BAFF and APRIL are cytokines belonging to the TNF-family. BAFF can bind to three different receptors: the BAFF receptor (BAFF-R), TACI, and (weakly) to BCMA (B cell maturation antigen). APRIL binds to TACI and to BCMA. The most important cellular effects of binding of BAFF and APRIL to the various receptors are indicated.

saccharides from encapsulated bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae* type b and *Neisseria meningitidis*. Neither processing and antigen presentation in the context of MHC class II molecules nor specific T lymphocyte activation has been demonstrated for polysaccharide molecules. This means that, because CD40/CD40L interaction is highly unlikely to take place in the case of T cell-independent antigens, polysaccharide-specific B lymphocytes should receive alternative costimulatory signals. Indirect evidence points towards a role for the CD19/CD21 receptor complex in this respect [9]. CD19 is a 95-kDa glycoprotein of the immunoglobulin superfamily that is expressed throughout B lymphocyte development. A specific ligand for this molecule has not been identified, although purified CD19 protein does bind to bone marrow stromal cells. Activation of CD19 by specific antibodies provides a costimulatory signal for B lymphocyte activation through the BCR. Indeed, the cytoplasmic domain of the CD19 molecule contains cell-signaling motifs. On mature B lymphocytes, CD19 is expressed in a molecular complex that includes CD21, the TAPA-1 protein (CD89) and the Leu-13 molecule. The prevailing model is that, in this complex, CD19 acts as the signal-transducing moiety for CD21. CD21 is a 145-kDa glycoprotein of the complement receptor family, which is expressed on mature B lymphocytes (and also on follicular dendritic cells and at a low level on a subpopulation of T cells). CD21 is the receptor for the complement component C3 split products iC3b, C3dg and C3d. CD21 also serves as the cellular receptor for the Epstein-Barr virus, and as an IFN-α

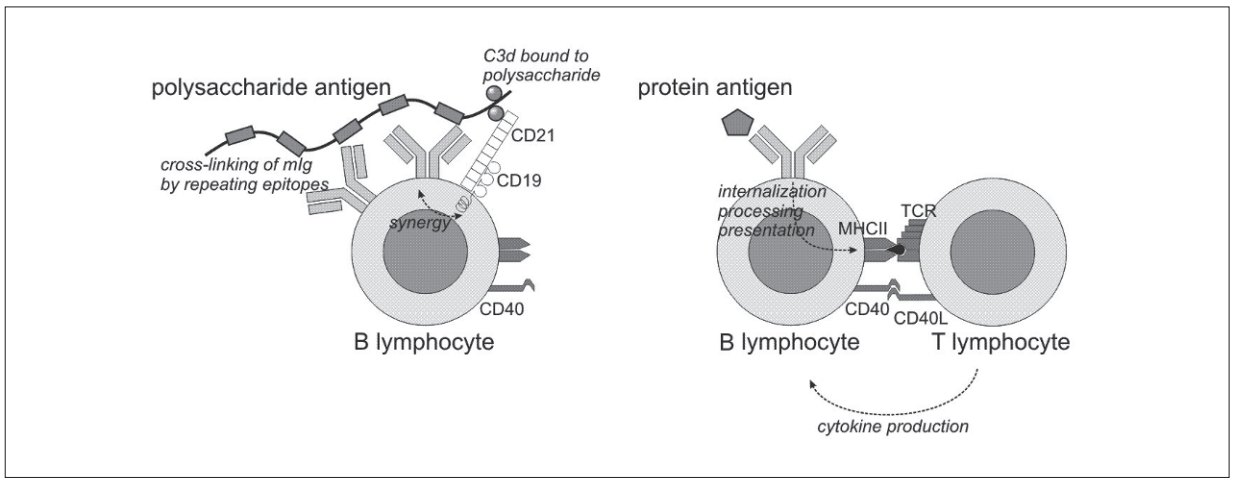

Figure 8. B lymphocyte activation by T cell-independent and by T cell-dependent antigens
Left panel: A polysaccharide antigen with repeating epitopes cross-links mIg on the surface of the B lymphocyte. Deposited C3d is bound by CD21, which provides a synergistic signal for B lymphocyte activation. Right panel: A protein antigen is bound and internalized by mIg. Following intracellular processing, peptide fragments are expressed in MHC class II molecules. Specific T lymphocytes recognize these peptides in the context of MHC, become activated and express CD40L. Upon interaction of CD40L with CD40, cytokine production is initiated (see Fig. 5 for more details).

receptor. Furthermore, CD21 can interact with CD23. The (chemical) coupling of C3d to protein antigens lowers the threshold for antibody induction 100–1000-fold [10]. Bacterial polysaccharides, through the alternative pathway of complement activation, can generate C3 split products, which become deposited on the polysaccharide. Natural complexes of polysaccharide and C3d, thus formed, can cross-link mIg and CD21 on polysaccharide-specific B lymphocytes. This mechanism may bypass the need for engagement of CD40/CD40L in B lymphocyte activation (Fig. 8). Compatible with this mechanism is the finding that children up to the age of 2 years who are unable to respond to polysaccharide antigens have a reduced expression of CD21 on B lymphocytes [11].

Primary and secondary antibody response

The first contact of the immune system with a given antigen will induce what is called a primary (antibody) response. B lymphocytes become activated and differentiate into plasma cells (along routes described above). Plasma cells are highly differentiated cells, which maximally produce 10^4 antibody molecules per second, equaling 40% of the total protein-synthesizing capacity of the cell. The lifetime of a plasma cell is 3–4 days in the initial phase of the antibody response. These plasma cells are called short-lived plasma cells. A second contact with the same (protein) antigen elicits a secondary antibody response, which is produced by long-lived plasma cells and differs in a number of aspects from a primary response [12]. The latency period (time between contact with antigen and start of antibody production) is shorter in a secondary response (Fig. 9), and antibody levels attained are much higher (one to two orders of magnitude). Whereas during a primary antibody response predominantly IgM, and to a lesser extent IgG antibodies are produced, IgG, IgA and IgE antibodies are the major classes during a secondary response. The affinity of antibodies produced increases during the response; there may be a 100–1000-fold difference in affinity between antibo-

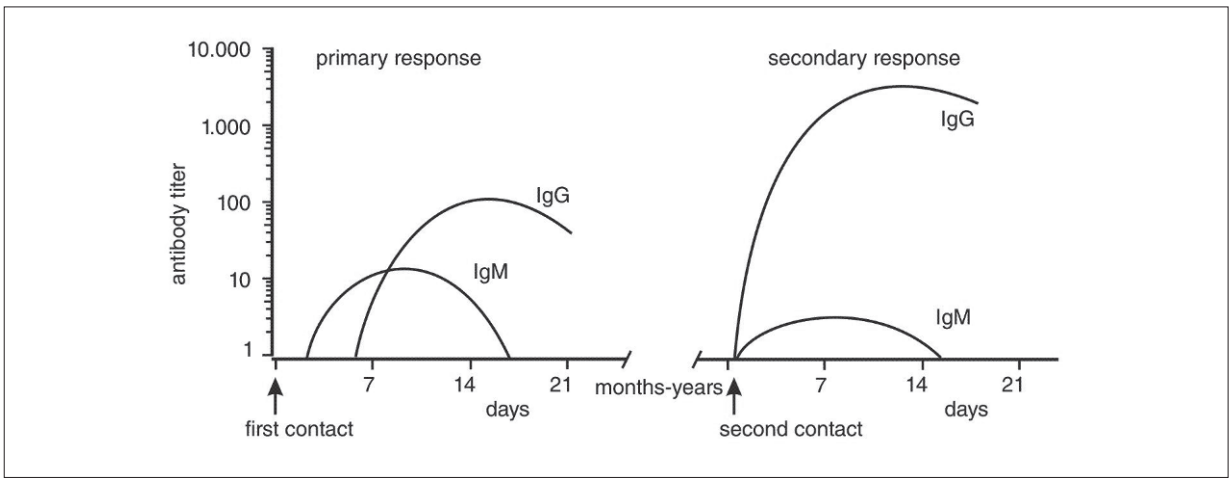

FIGURE 9

The response triggered by the first encounter with a given antigen is called the primary antibody response. During this response IgM appears first, followed by IgG. The most prevalent class of antibody, IgG is produced when a particular antigen is encountered again. This response is called the secondary antibody response. It is faster and results in higher antibody titers than the primary antibody response.

DIES produced by the short-lived plasma cells at the start of a primary response and at the end of a secondary response. This process (AFFINITY MATURATION) is the combined effect of somatic hyper-mutation of CDR1 and CDR2 regions during B lymphocyte proliferation and the selection of the B LYMPHOCYTES with the highest AFFINITY (as described earlier).

A primary ANTIBODY response takes place in FOLLICLES, the marginal zone of spleen and lymph nodes. During a secondary response, BONE MARROW is the major site of ANTIBODY production. Long-lived plasma cells in the BONE MARROW can survive for at least 90 days in the absence of cell division. ANTIBODIES that are secreted in mucosal tissue of the respiratory and gastrointestinal tract are produced locally by the bronchus-associated lymphoid tissue (BALT) and gut-associated lymphoid tissue (GALT), respectively (Fig. 10).

During the primary immune response, some of the antigen-specific B LYMPHOCYTES do not differentiate into plasma cells but into so-called memory B LYMPHOCYTES. Whether an activated B lymphocyte differentiates into a plasma cell or into a memory B lymphocyte is largely determined by the relative expression of transcription factors: Blimp-1 is the master regulator for plasma cell generation, while Bcl-6 promotes memory B lymphocyte formation. Bcl-6 suppresses APOPTOSIS genes, which contribute to the longevity of memory B LYMPHOCYTES [13]. Naïve and memory B LYMPHOCYTES differ in expression of the ISOTYPES of mIg and the AFFINITY of mIg. Naïve primary B LYMPHOCYTES express mIgM and mIgD; memory B LYMPHOCYTES have lost mIgD and express mIgG or mIgA, with or without mIgM. Adult circulating B LYMPHOCYTES can be separated into three subpopulations on the basis of CD27 and mIgD expression: IgD^+CD27^- naïve B LYMPHOCYTES, IgD^+CD27^+ non-switched memory B LYMPHOCYTES, and IgD^-CD27^+ memory B LYMPHOCYTES ([14]; see also Fig. 11). Because of the AFFINITY MATURATION described above, the AFFINITY of mIg for antigen on memory B LYMPHOCYTES is higher than on primary B LYMPHOCYTES.

All characteristics of primary and secondary ANTIBODY responses, as described above, hold true only for protein ANTIGENS. For polysaccharide ANTI-

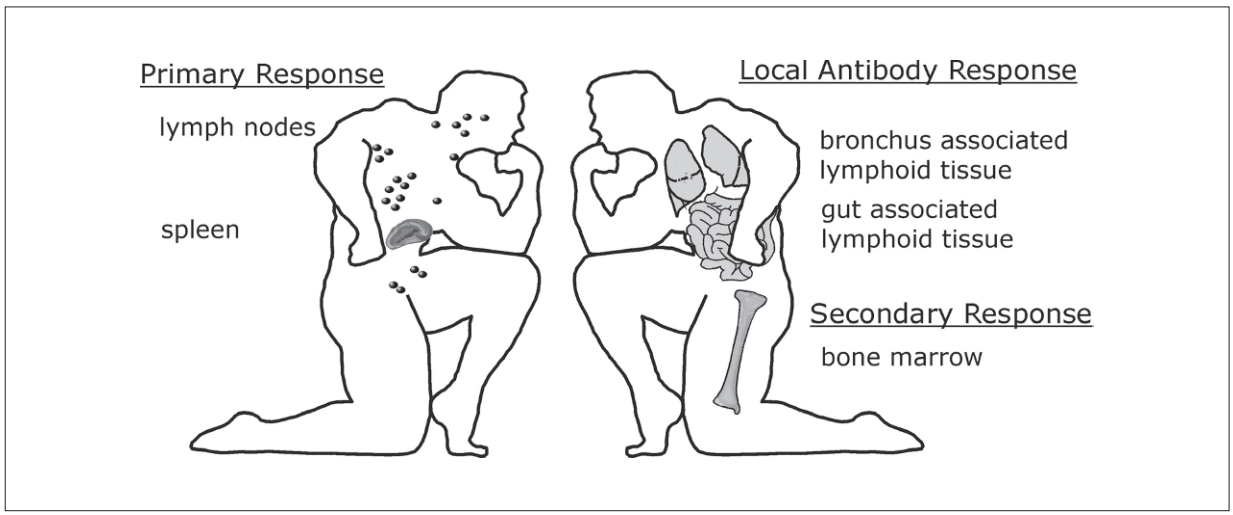

Figure 10. Sites of antibody production

gens, a second contact with antigen induces an antibody response that is identical in kinetics and magnitude to the primary response. Affinity maturation does not occur, and isotype distribution of antibodies does not change. Moreover, anti-polysaccharide antibodies use a restricted number of V_H and V_L genes, whereas anti-protein antibodies are more heterogeneous. Finally, IgG anti-protein antibodies to the vast majority of antigens are of the IgG1 subclass; IgG anti-polysaccharide antibodies in adult individuals are predominantly IgG2.

Biological functions of antibodies

The biological functions of antibodies are exerted by the different parts of the molecule: binding of antigen is carried out by variable V_H and V_L domains, while effector functions are mediated by the constant domains (in particular C_{H2} and/or C_{H3} domains). In a few cases, antibody molecules can have a direct biological effect by binding to antigen. The bacterial toxins mentioned at the beginning of this chapter are neutralized when bound by antibodies. Anti-viral antibodies can reduce the spread of virus particles through the body. For all other functions of antibodies, interaction of the antibody molecule with other effector mechanisms is required. Clearance of immune complexes and antibody-opsonized microorganisms depends on the functional integrity of the Fc part of the antibody molecule. The Fc part can either interact with soluble biologically active molecules, such as the complement system (see chapter A6) or bind to Fc receptors that are expressed on a variety of cells of the immune system [15]. Fc receptors expressed on monocytes, macrophages and granulocytes are essential for phagocytosis of immune complexes and opsonized microorganisms. Fc receptors for IgG (Fcγ receptors) are expressed on monocytes and macrophages; neutrophilic granulocytes also express receptors for IgA (Fcα receptors). Depending on the class of antibodies in an immune complex, the complement system becomes more or less efficiently activated. This will enhance phagocytosis by monocytes, macrophages and granulocytes, since these cells also express complement receptors in addition to Fc receptors.

Fc receptors for IgE (Fcε receptor) are primarily expressed by mast cells. In allergic individuals, Fcε receptors have constitutively bound IgE; exposure to allergens causes cross-linking of Fcε receptors, resulting in mast cell degranulation and histamine release (see chapter A9). Apart from phagocytosis and

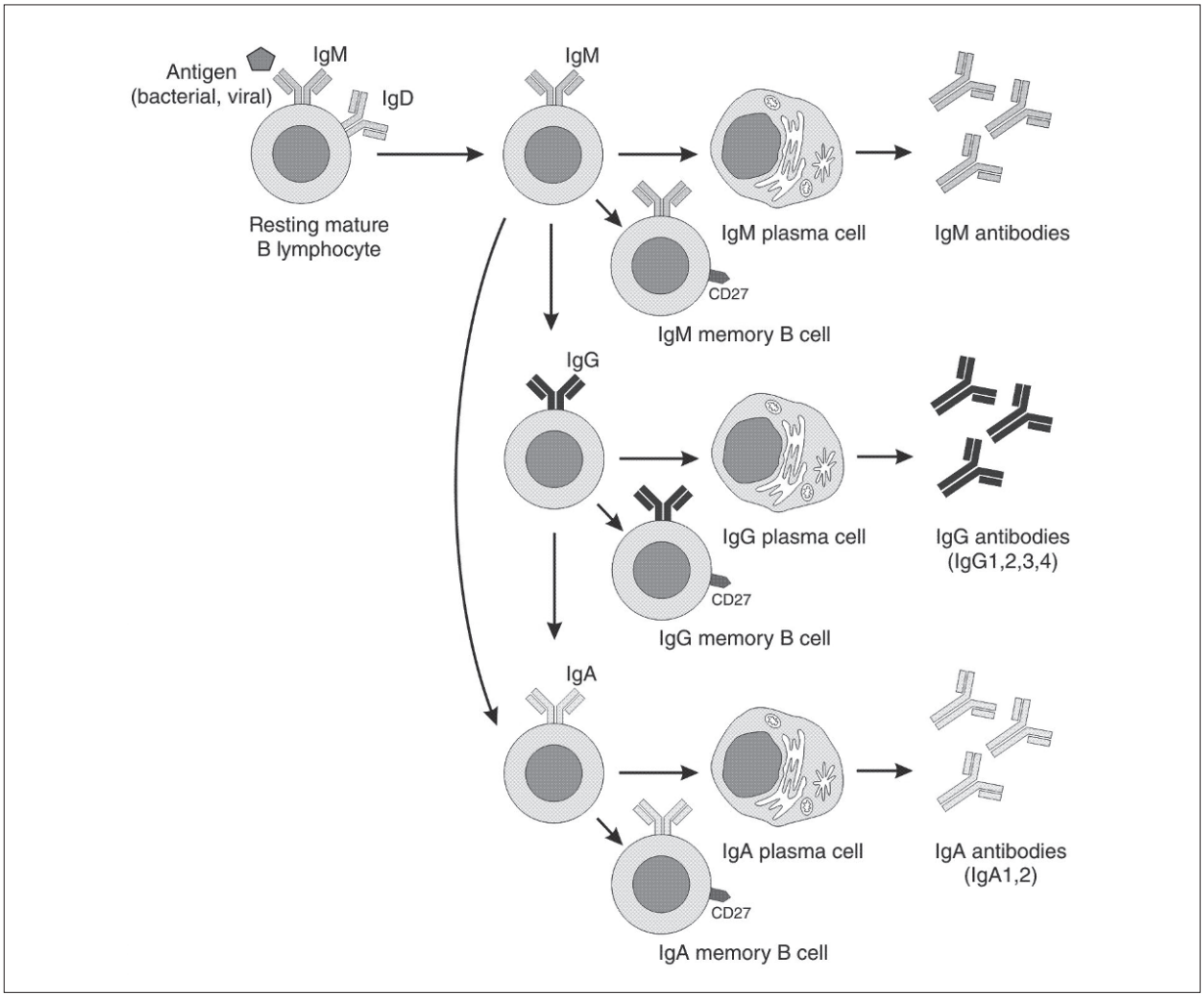

Figure 11. Generation of memory B lymphocytes

degranulation, Fc receptors also mediate cytotoxicity in a process called antibody-dependent cellular cytotoxicity (ADCC). Target-cells (e.g., tumor cells) to which antibodies are bound can be recognized by Fc receptors expressed on cells with cytotoxic potential. The killing process itself is complement independent. Monocytes, neutrophilic and eosinophilic granulocytes and natural killer (NK) cells display ADCC activity. ADCC can be a mechanism for removal of tumor cells and has been implicated in tissue damage that occurs in autoimmune diseases.

The Fcγ receptor expressed on B lymphocytes (FcγIIb) plays a role in down-regulation of B lymphocyte activation. When high IgG antibody concentrations are reached during an immune response, antigen-IgG complexes will be formed, which can cross-link the BCR and FcγIIb on the surface of the B lymphocyte (see also Fig. 12). The cytoplasmic domain of FcγIIb contains a YSLL motif, which has been termed ITIM for immunoreceptor tyrosine-based inhibitory motif. Tyrosine phosphorylation of this motif causes the association of a protein

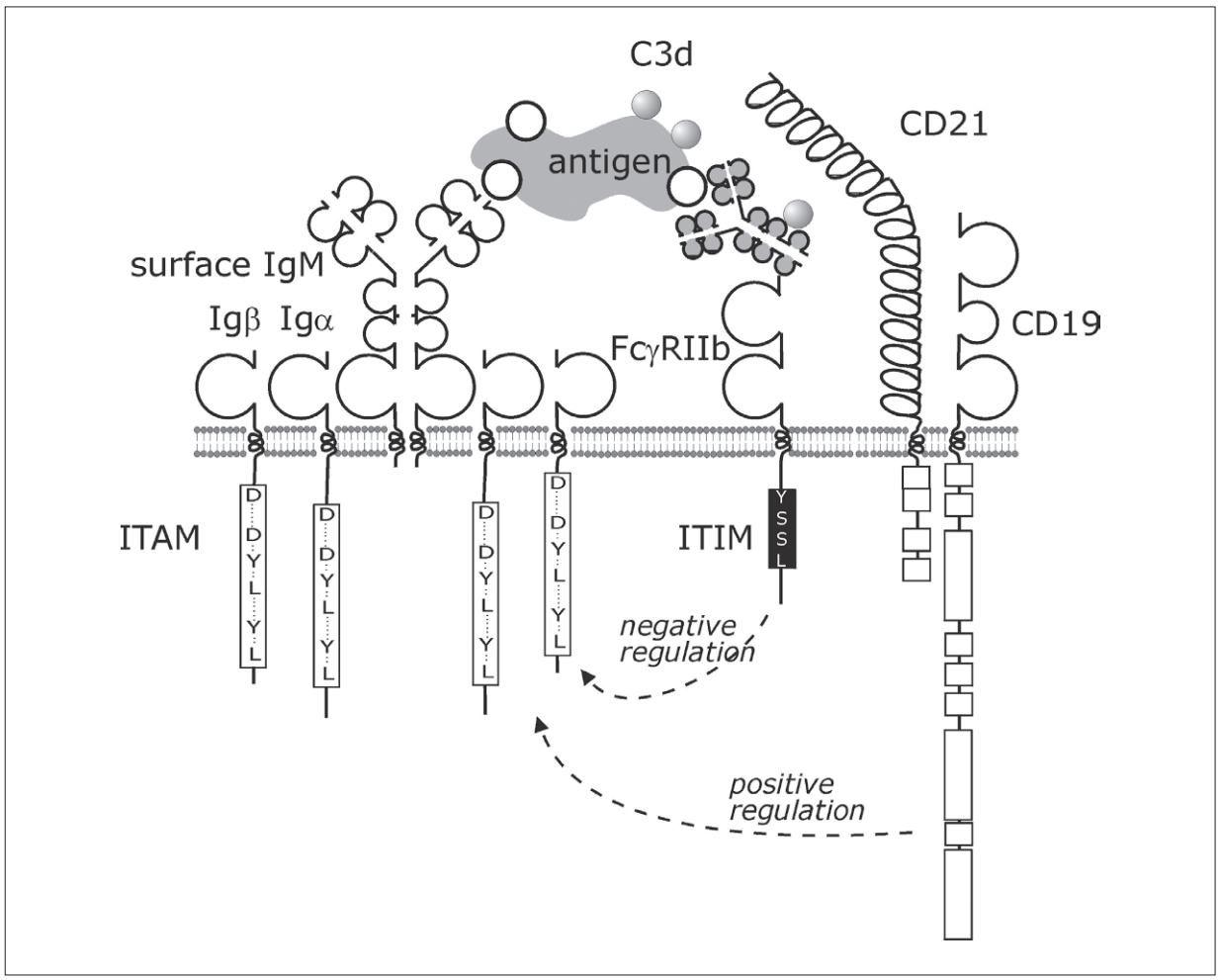

Figure 12. Regulation of BCR activation by co-receptors

Ovals in membrane immunoglobulin (surface IgM), Igα, Igβ, FcγRIIb and CD19 indicate homologous domains of immunoglobulin superfamily members. The cytoplasmic domains of Igα and Igβ, contain the ITAM. The Fcγ receptor IIb binds the Fc part of IgG antibodies in immune complexes. The cytoplasmic domain of FcγRIIb contains the immunoreceptor tyrosinase-based inhibitory motif (ITIM) YSLL. CD21 (type 2 complement receptor) binds C3d deposited on the antigen or on IgG antibodies. The cytoplasmic domain of CD19 has a positive regulatory effect on signaling through mIg (see text for further explanation).

tyrosine phosphatase. When brought in close proximity to ITIMs, this enzyme causes tyrosine dephosphorylation and therefore inhibits BCR signaling. This mechanism, by which IgG ANTIBODIES interact with FcγIIb and co-cross-link with the BCR, is an example of active down-regulation of the ANTIBODY response. There are other examples of cell surface RECEPTORS with either intrinsic (CD45) or associated protein tyrosine phosphatase activity (CD22), but their cellular ligands, and therefore their role in regulation of B lymphocyte activation, are at present unknown.

Clinical relevance and future prospects

The integrity of the humoral IMMUNE SYSTEM is crucial for host defense against bacterial and certain viral infections. Inborn or acquired deficiencies in humoral immunity result in increased susceptibility to potentially life-threatening infections. A dysregulated humoral IMMUNE SYSTEM may result in conditions such as ALLERGY. These and other clinical aspects are discussed elsewhere in this volume (see chapters A9, A10 and C3).

Most of what is known about the cellular and molecular aspects of B lymphocyte activation and regulation has been gathered from experiments performed during the last few decades. Detailed knowledge of the mechanisms governing the regulation of expression of cell surface RECEPTORS and signaling mechanisms allows pharmacological intervention in ANTIBODY-mediated immunity. Intervention is possible at three levels: outside the B lymphocyte, at the cell surface and intracellularly. The coupling of polysaccharides to protein carriers changes the nature of the anti-polysaccharide ANTIBODY response from being T cell independent into T cell dependent. These polysaccharide-protein conjugate vaccines bypass the selective unresponsiveness to T cell-independent ANTIGENS early in life, and thus constitute novel and effective tools in prevention of infectious diseases. Conjugate vaccines for *Haemophilus influenzae* type b and *Streptococcus pneumoniae* have been shown to prevent invasive diseases in otherwise susceptible populations. In case of uncontrolled proliferation of B LYMPHOCYTES (B cell lymphoma) or unwanted autoantibody production (AUTOIMMUNE DISEASES), B LYMPHOCYTES can be eliminated by MONOCLONAL ANTIBODIES directed to the CD20 molecule (see chapters C3 and C15). These reagents (BIOLOGICALS) are powerful tools in managing these abnormalities of the humoral IMMUNE SYSTEM [16]. Care must be taken to reduce the negative side effects associated with complete depletion of B LYMPHOCYTES

Summary

ANTIBODIES are produced by B LYMPHOCYTES. Upon binding of antigen to mIg, the B lymphocyte becomes activated and differentiates into an ANTIBODY-producing plasma cell. mIg is part of the B cell RECEPTOR complex, in which Igα and Igβ proteins are signal transduction molecules. Key elements in B lymphocyte signaling are phosphorylation of tyrosine residues within the ITAM of Igα and Igβ and subsequent activation of a series of cytoplasmic tyrosine kinases. Apart from triggering of mIg, full B lymphocyte activation requires a number of additional molecular interactions, which require cognate cellular interaction with T LYMPHOCYTES and/or MONOCYTES (T cell-dependent B cell activation). The binding of CD40 to the B lymphocyte and CD40L to activated T LYMPHOCYTES is an example of these costimulatory interactions. Furthermore, CYTOKINES, such as IL-4 and IL-5, promote B lymphocyte proliferation and differentiation. For B lymphocyte activation by polysaccharide ANTIGENS, T LYMPHOCYTES are not required. COSTIMULATION in this case is provided by CD21, the complement RECEPTOR on B LYMPHOCYTES that is activated by C3d, bound to the polysaccharide.

A primary ANTIBODY response starts with production of IgM ANTIBODIES. During a primary response, CLASS SWITCHING to IgG and IgA ANTIBODIES takes place. Proliferating B LYMPHOCYTES undergo somatic hypermutation, which can result in ANTIBODIES with a higher AFFINITY. During a primary response, a portion of the B LYMPHOCYTES differentiate into long-lived memory B LYMPHOCYTES (and express CD27). In a secondary response, the expanded CLONE of memory B LYMPHOCYTES reacts with a short latency period and high ANTIBODY production.

Upon interaction with antigen, ANTIBODIES exert a variety of biological functions: (1) they directly neutralize bacterial toxins; (2) initiate complement activation, which, in the case of a cellular antigen, results in cell lysis; (3) augment PHAGOCYTOSIS after interaction with Fc RECEPTORS; and (4) initiate ADCC.

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Recommended websites

http://www.antibodyresource.com/educational.html (portal with many links to relevant websites)

http://www.molbiol.ox.ac.uk/www/pathology/tig/new1/mabth.html (on therapeutic use of antibodies)

http://people.ku.edu/%7Ejbrown/antibody.html (what the heck is an antibody)

http://www.primaryimmune.org/ (many links to primary immunodeficiencies, including B lymphocyte deficiencies)

http://www.nature.com/nri/info/info_links.html (page from *Nature Reviews in Immunology* with a great number of links to various aspects of immunology)

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Dendritic cells

Geert R. Van Pottelberge, Ken R. Bracke and Guy G. Brusselle

Introduction

DENDRITIC CELLS (DCs) were first described in human skin by Paul Langerhans in 1868 [1]. Their function remained unknown until 1973, when it was shown that DCs have a unique immunological key function: they are capable of presenting ANTIGENS to LYMPHOCYTES to induce and regulate adaptive immune responses [2]. Hence, DCs are called professional antigen-presenting cells. Only two other cell types have similar antigen-presenting functions: MACROPHAGES (which are closely related to certain subsets of DCs) and B cells. However, DCs are by far the most powerful antigen-presenting cells in the IMMUNE SYSTEM.

DCs stand guard in the skin, the mucosal surfaces (respiratory, gastrointestinal and urogenital MUCOSA) and in the organs where they continuously sense for danger signals and capture ANTIGENS (Fig. 1). DCs then migrate towards draining LYMPH NODES, while integrating the information of danger signals with the processed antigen. In the lymph nodes, DCs present the processed antigen on MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) molecules and make contact with naïve LYMPHOCYTES. At this point, the DC is capable of selecting T cells with an antigen-matched T CELL RECEPTOR for clonal expansion. In addition to stimulation of clonal proliferation of T cells, DCs can direct and control the nature and extent of the T cell response. DCs thus play a central role in the initiation of primary adaptive immune responses and the enhancement of secondary immune responses. In this way, DCs form the crucial link between innate and ADAPTIVE IMMUNITY [3, 4].

Biology of the DC

DCs originate from hematopoietic precursors (see chapter A1). In general, there are two major distinct subsets of DCs: (1) myeloid DENDRITIC CELLS, which originate from myeloid progenitors, and which can directly differentiate from MONOCYTES, and (2) plasmacytoid DENDRITIC CELLS, which originate from lymphoid precursors and have a plasma cell-like morphology. Myeloid and plasmacytoid DCs express different surface markers, as shown in Figure 2. Identification of DCs is complicated due to the shared expression of so-called DC surface markers with other white blood cells. Therefore, identifying DC subsets ideally requires a combination of different surface markers, making multicolor FLOW CYTOMETRY the best technique to approach this issue (see chapter B3). Using this technique, DCs are generally identified within the low AUTOFLUORESCENT, lineage-negative, HLA-DR-positive cell population with the expression of the myeloid surface marker CD11c as the discriminating factor between myeloid ($CD11c^+$) and plasmacytoid ($CD11c^-$) DCs [5, 6].

Circulating blood DCs express the blood DC ANTIGENS (BDCA), subdividing the blood DCs into different subsets [7]. However, only BDCA-2 is specific for a DC subset, as the remaining BDCA markers are also expressed on other white blood cells.

Myeloid DCs that have left the blood circulation and have entered the mucosal area (tissue resident DCS) can express additional surface markers such as DC-SIGN (CD209; DC-specific intracellular ADHESION MOLECULE 3-grabbing non-integrin), CD163 and Factor XIIIa. These immature myeloid DCs are called "interstitial DCs" and are closely related to MONOCYTES and MACROPHAGES. Interstitial-type DCs are mainly located

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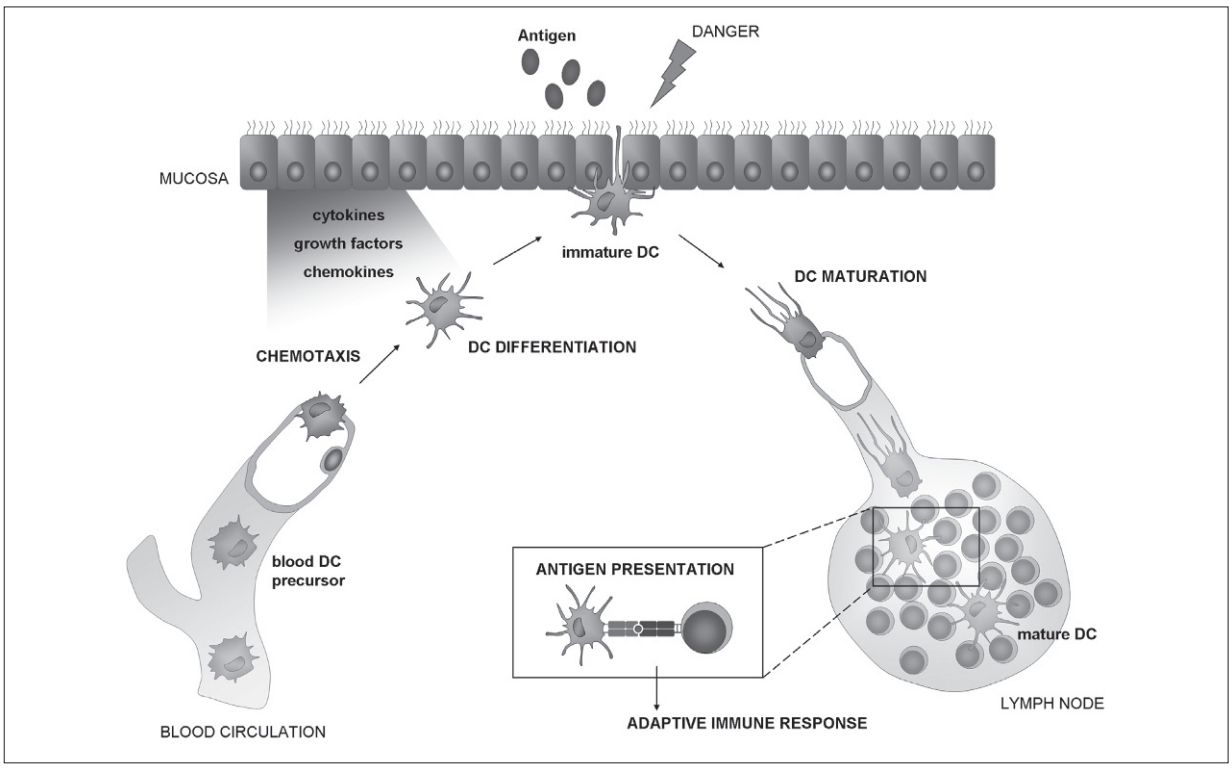

Figure 1. The dendritic cell (DC) sentinel paradigm

DC precursors migrate from the blood stream into the mucosa and differentiate to immature DCs under the influence of cytokines and growth factors, mainly released by epithelial cells. DCs capture antigens and sense for danger signals in the mucosa. They then mature and migrate towards draining lymph nodes where they interact with naïve lymphocytes.

in the lamina propria and adventitia of the mucosal areas and in the dermis of the skin. DC-SIGN$^+$ DCs can be generated from CD14$^+$ monocytes *in vitro* in the presence of GM-CSF and IL-4 [8].

A second subgroup of tissue-residing DC expresses langerin (CD207), which is a specific marker for a special myeloid DC subset: the Langerhans cell. Langerhans cells are well characterized in the skin with the expression of tennis racket-shaped organelles (known as Birbeck granules) on electron microscopy. Analogue langerin-positive, Birbeck granule-positive Langerhans-type DCs have also been reported in the respiratory and genital mucosa. These Langerhans-type DCs are mainly located in the epidermis and the epithelium of the mucosa. Langerhans-type DCs can also express CD1a at their cell surface. Monocytes can differentiate towards Langerhans-type DCs *in vitro* in the presence of GM-CSF and IL-4 combined with TGF-β and TNF-α [9, 10].

Plasmacytoid DCs can also be found in mucosal areas, be it in far lower numbers than myeloid DCs. They are mainly located in lymphoid aggregates and in secondary lymphoid organs, in close contact with the T cell zone. These immature plasmacytoid DCs are capable of directly migrating from the blood circulation to the secondary lymphoid organs through specialized high endothelial venules [11].

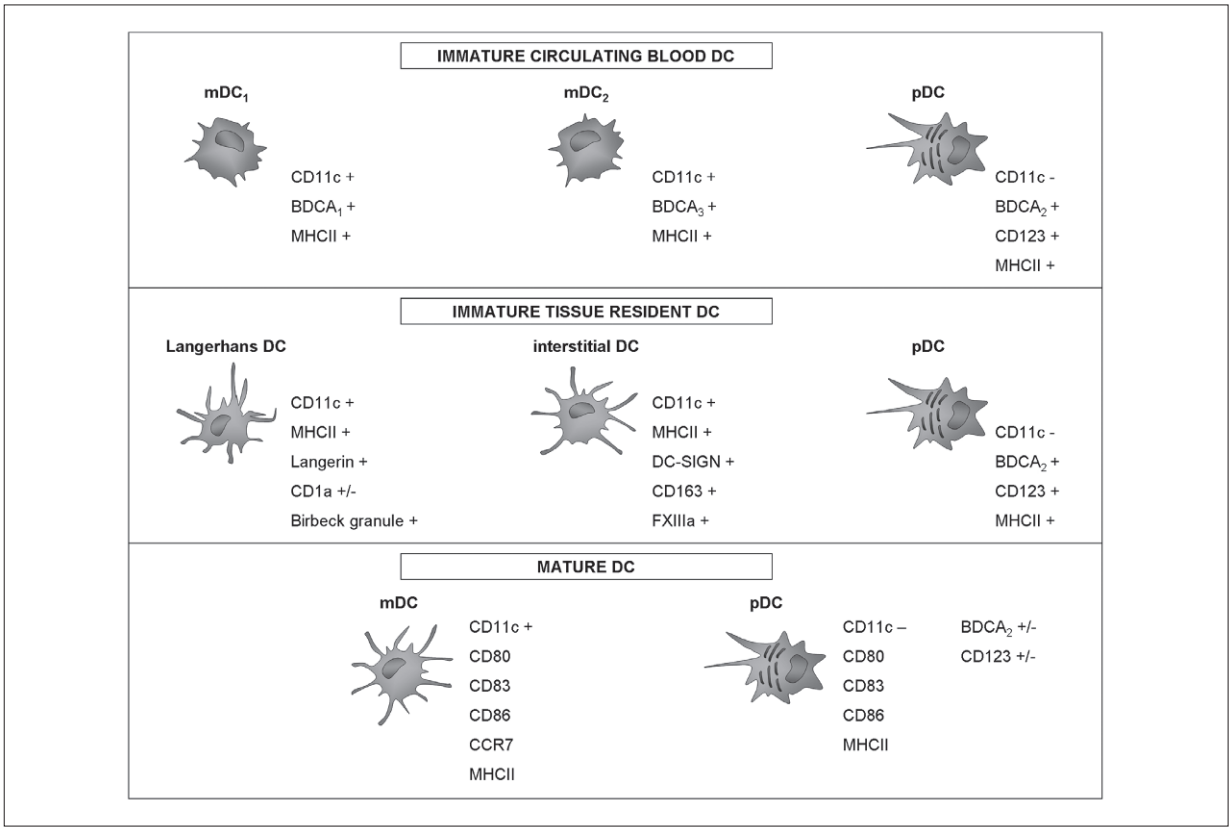

FIGURE 2. DC SUBSETS

DCs can be divided into subsets that express different surface molecules. DC phenotype differs between circulating blood DCs and tissue resident DCs. Mature DCs express specific maturation markers. mDC1, myeloid DC type 1; mDC2, myeloid DC type 2; pDC, plasmacytoid DC; BDCA, blood DC antigen

Under certain stimuli (pathogen-associated molecular patterns, PAMP, danger signals released by dying cells or a mixture of certain CYTOKINES), myeloid and plasmacytoid DCs will undergo a maturation process. DC maturation is associated with several coordinated events, including (i) loss of endocytic/phagocytic RECEPTORS and diminished antigen capture, (ii) change in cellular morphology with development of large cytoplasmic protrusions (dendrites), (iii) translocation of MHC class II antigen-presenting molecules to the cell surface; (iv) up-regulation of COSTIMULATORY MOLECULES, and (v) expression of lymphoid homing chemokine RECEPTOR CCR-7. These mature DCs migrate towards the secondary LYMPHOID ORGANS (lymph nodes and spleen), where they make contact with T cells to steer the adaptive immune response.

Mechanisms and pathways

Innate immune functions of DCs

Immature DCs form a strategically located network in or just beneath the epithelium of the mucosal areas and often reach out into the lumen with their protrusions to sense for danger signals. For this purpose,

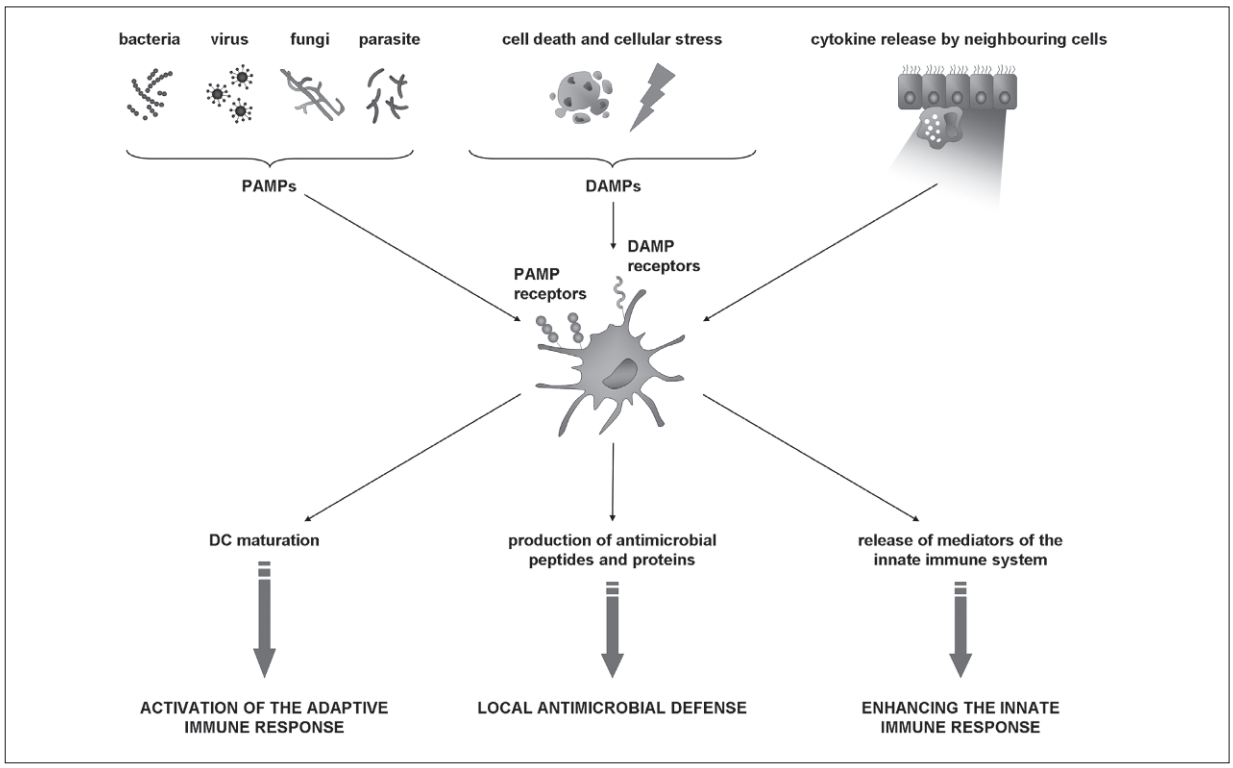

Figure 3. Innate and adaptive immune functions of DCs

DCs participate in the innate immune response by pattern recognition of microbial signals and recognition of cellular stress and damage. Upon activation by these signals, DC mature and activate the adaptive immune response. In addition, these DC are also capable of producing antimicrobial peptides, contributing to first-line antimicrobial defense. Finally, DC produce mediators that attract other immune cells, enhancing the innate immune response. PAMP, pathogen-associated molecular pattern; DAMP, damage associated molecular pattern; IL, interleukin; TNF-α, tumor necrosis factor alpha

DCs are equipped with various RECEPTORS capable of detecting PATHOGEN-ASSOCIATED MOLECULAR PATTERNS (PAMPs) and damage-associated molecular patterns (DAMPs) (Fig. 3). Signaling through these 'danger RECEPTORS' activates the process of maturation and enhances antigen processing. Activation of DCs is an essential first step for induction of ADAPTIVE IMMUNITY [12].

Among the different DC subsets, specialization in danger recognition is present: plasmacytoid DCs are specialized in recognition of viral PAMPS through TOLL-LIKE RECEPTORS (TLR)-7 and 9, triggering the production of interferon-α, which is the cornerstone of the early antiviral defense mechanism. In contrast, myeloid DCs are capable of detecting both bacterial and viral PAMPS through different TLRs, with a differential expression of TLR-2 and 4 between interstitial-type and Langerhans-type DCs. Microbial patterns are also detected by DCs through the nucleotide-binding and oligomerization domain (NOD)-like RECEPTORS and retinoid acid inducible gene (RIG)-like RECEPTORS.

Other pathogen-associated PATTERN RECOGNITION RECEPTORS expressed on DCs are the C-type LECTIN

Table 1. Relative expression of Toll–like Receptors (TLR) on different dendritic cell subsets

	Blood circulation			Skin DC	
	Circulating myeloid DC	Monocytes	Plasmacytoid DC	Langerhans DC	Interstitial DC
TLR1	–	+	+	+/–	++
TLR2	+++	+++	–	+/–	++
TLR3	–	+	–	++	+
TLR4	+	+++	–	–	+++
TLR5	+++	+	–	–	++
TLR6	+	+	+	+	++
TLR7	–	–	+++	++	+
TLR8	+	+	–	–	++
TLR9	–	–	+++	++	+
TLR10	–	–	+	++	+

Adapted from [12, 34].

receptors (such as DC-SIGN and langerin), which are capable of binding carbohydrate structures present on pathogens. These C-type lectins are involved in the internalization process of pathogens, but are also crucial for interactions of the DC with the extracellular matrix.

Another type of 'danger receptors' are the DAMP receptors, such as receptor for advanced glycation end products (RAGE), and purinergic receptors, which can detect molecules released by damaged or dying cells. Some PAMP receptors such as TLRs also function as DAMP receptors, indicating that immune responses against pathogens and cell damage go side by side.

Activation of DCs by 'danger receptors' will trigger the production of proinflammatory cytokines and chemokines such as TNF-α, IL-1, IL-6, IL-8 and IL-12. In addition, DCs will release a series of innate immune molecules such as pentraxins and neuropeptides *in situ* [13, 14], contributing to the first line of defense.

Antigen sampling, processing and presentation by DCs

T cell immunity essentially relies on recognition of denatured, unfolded, sequential determinants of proteins. Therefore, proteins need to be processed by DCs in order to be recognized by T cells. In addition, correct presentation of these processed peptides needs to occur in the groove of surface-expressed MHC class molecules to be recognized by a specific T cell receptor (see chapter A2). A complex intracellular machinery in the DC is involved in this process of antigen capture, processing and presentation [15] (Fig. 4).

MHC class II antigen presentation

The MHC class II antigen presentation pathway is essentially restricted to professional antigen-presenting cells and does not occur in other cell types (except for epithelial cells in the thymus and

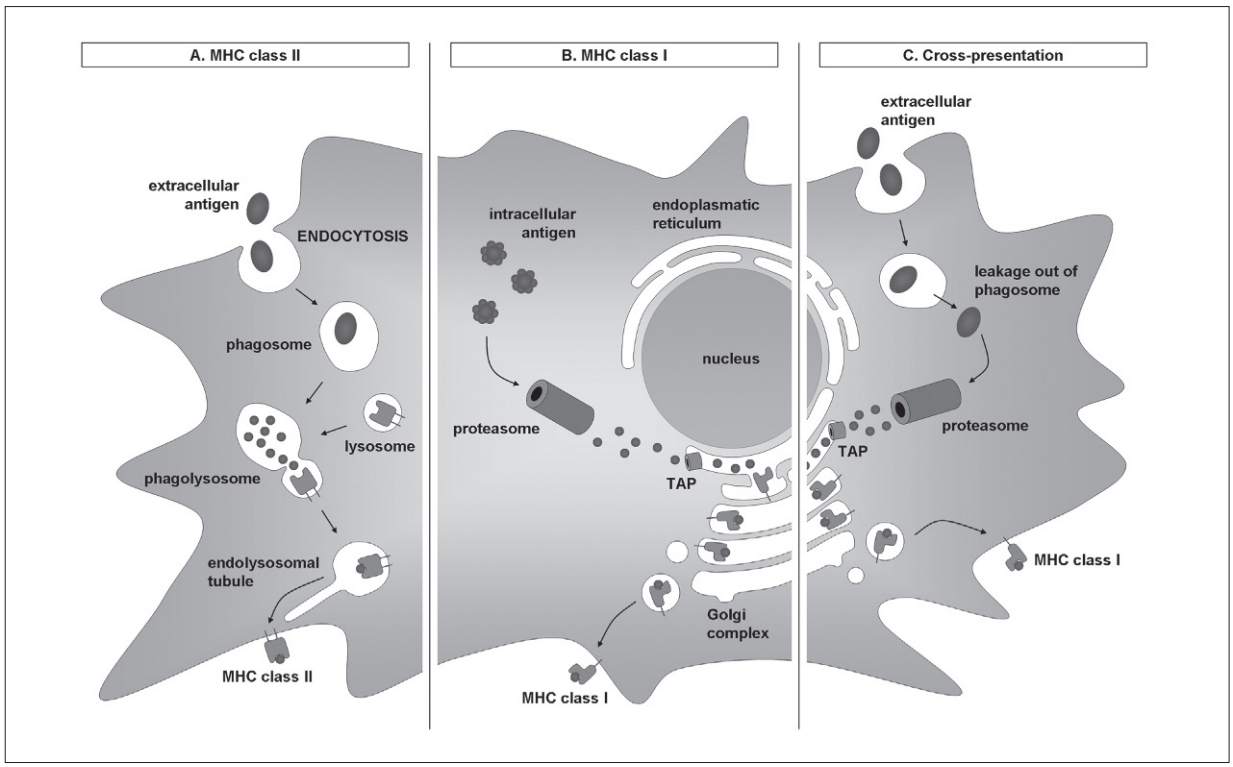

Figure 4. Antigen processing and presentation by DCs

(A) Extracellular antigens are presented on major histocompatibility class (MHC) class II molecules. These antigens are phagocytosed by the DC, creating a phagosome. By fusion with a lysosome (which brings along the MHC class II molecule), the phagosome becomes a phagolysosome. In this phagolysosome, the antigen is degraded to peptide fragments, which are loaded onto the MHC class II molecule. These antigen-MHC complexes are then transported to the cell membrane though an endolysosomal tubule. (B) Intracellular cytoplasmic antigens are presented by MHC class I molecules after processing by the proteasome in the cytosol and uptake in the endoplasmic reticulum through TAP (transporter associated with antigen processing). The MHC class I molecules loaded with processed antigen are then transported to the cell membrane by the Golgi apparatus. (C) Cross-presentation is the presentation of extracellular antigens by the MHC class I pathway. Antigens presumably leak out of the phagosome into the cytosol where they are further processed by the proteasome.

microglia in the brain). The goal of this pathway is to sample the extracellular compartment and present the ANTIGENS to $CD4^+$ T cells. When a micro-organism is phagocytosed or particulate matter is ingested by macropinocytosis, TLRs become activated resulting in maturation of the PHAGOSOME, which will then merge with a lysosome to become a phagolysosome. Lysosomal proteases degrade the microbial proteins to peptide fragments. These peptides will be loaded onto MHC class II molecules, supplied by the lysosomes. Peptide-loaded MHC class II molecules are then transported towards the cell surface by an endolysosomal tubule.

MHC class I antigen presentation

The goal of MHC class I antigen presentation is to report intracellular events (such as viral infection,

intracellular bacteria or cellular transformation) to the adaptive IMMUNE SYSTEM to activate $CD8^+$ T cells. Proteins in the cytosol (e.g., viral proteins or misfolded proteins) are tagged for degradation by the proteasome. Small peptide fragments are then transported to the endoplasmic reticulum by the transporter associated with antigen processing (TAP) complex. The peptides are loaded onto the MHC class I molecules. These MHC-peptide complexes are then transported through the Golgi complex and arrive at the cell surface. This pathway of antigen presentation is not restricted to antigen-presenting cells but occurs essentially in all cells (except for red blood cells). Virus-infected cells can be eliminated by CYTOTOXIC T cells through MHC class I presentation.

Cross-presentation

DCs have the capability to take up ANTIGENS from the extracellular environment and present them through MHC class I molecules, inducing a $CD8^+$ T cell response. This process is known as "cross-presentation" or "cross-priming" and is crucial for the initiation of immune responses to viruses that do not directly infect antigen-presenting cells.

DC–lymphocyte interaction: formation of the immunological synapse

DCs that have captured and processed antigen can undergo a process of maturation: apart from the presentation of the antigen-peptide on MHC molecules, they express co-stimulatory molecules to a certain extent, dependent on the degree of danger signals the DCs received. In general, mature DCs migrate towards the regional lymph nodes using the lymphoid homing chemokine RECEPTOR CCR-7. DCs then interact with naïve T LYMPHOCYTES, during which the MHC-antigen peptide complex is recognized by a specific T CELL RECEPTOR. This matched T cell is then activated by the COSTIMULATORY MOLECULES (CD40, CD80 and CD86) expressed on the DC. In addition, the DC provides a third signal consisting of CYTOKINES that can activate and steer the T cell proliferation and differentiation.

ImmatureDCs, which do not express COSTIMULATORY MOLECULES, can not induce clonal T cell proliferation and they are therefore considered to be tolerogenic. In contrast, mature myeloid DCs are strong inducers of T cell responses. A certain set of CYTOKINES and membrane-bound molecules of the DCs in the immunological synapse further steer the T cell differentiation towards a T helper (Th) 1, Th2, Th17 or regulatory T cell response (Fig. 5). In particular, mature Langerhans DCs are strong activators of cellular immune responses (especially proliferation of $CD8^+$ CYTOTOXIC T cells, presumably through the production of IL-15) [16]. In contrast, interstitial-type DCs are more specialized to induce humoral responses through direct and indirect activation of B cells [17].

Plasmacytoid DCs express lower levels of MHC class II molecules and COSTIMULATORY MOLECULES, and they are less efficient in antigen processing. Therefore, plasmacytoid DCs are not as potent in inducing the clonal proliferation of naïve T cells. Plasmacytoid DCs are known to have indoleamine 2,3 dioxygenase (IDO) enzyme activity that inhibits T cell proliferation. In addition, through ICOS-ICOS LIGAND interaction, plasmacytoid DCs are capable of generating regulatory T cells. Thus, plasmacytoid DCs are mainly involved in maintaining TOLERANCE [18, 19].

Pathophysiological relevance

As DCs have a key position, linking innate and adaptive immune responses, they play an important role in diverse pathological processes [20].

In infectious diseases, DCs are indispensable for the initiation of the adaptive immune response against the invading micro-organism. However, various micro-organisms try to evade the immunological response by altering DC function. *Coxiella burnetti*, *Salmonella typhi*, plasmodia, HIV, CMV and herpes simplex virus achieve this deception by blocking DC maturation. Certain pathogens (e.g. *Yersinia pestis* and *S. typhi*) selectively inject toxins into PHAGOCYTES such as DCs to destroy them, whereas certain viruses can induce APOPTOSIS of DC. Finally, microbes can also interfere with the controlling function of DCs, switching the T cell response away from the

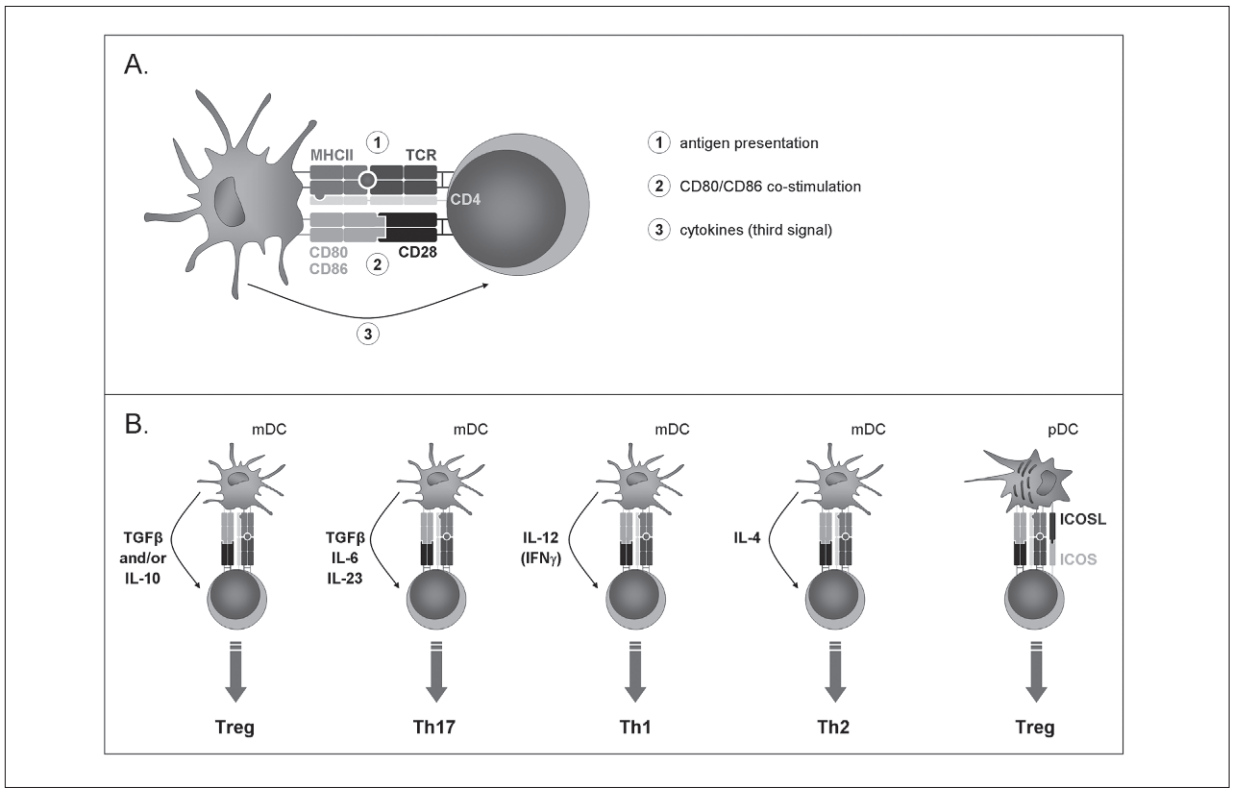

Figure 5. The immunological synapse

(A) DCs interact with naïve CD4+ T cells, steering the proliferation and differentiation of antigen-specific T cell clones. The processed antigen, presented on the MHC class II molecule, is recognized by the matched T cell receptor, flanked by CD4. A costimulatory signal from the mature DC is provided through CD80 and CD86, each interacting with a CD28 molecule on the T cell. Finally, a third signal is provided by the production of cytokines by the DC. (B) Depending on the third signal produced by a DC, CD4 T cell differentiation is skewed towards a Th1, Th2, Th17 or regulatory T cell response. Plasmacytoid DCs can induce regulatory T cells by inducible costimulator (ICOS)-ICOS ligand interaction.

protective Th1 response towards a non-protective Th2 response. In the case of HIV infection, different subsets of mucosal DCs appear to have distinct roles in viral transmission and clearance [21].

The role of DCs has been well described in allergic diseases such as asthma [22], in which myeloid DCs are capable of inducing an adaptive Th2-skewed T cell proliferation directed against a harmless antigen. Importantly, allergens themselves often contain a danger signal that is needed to activate the DC [23]. In a mouse model, exposure to a harmless antigen led to Th2 cell sensitization and features of asthma when plasmacytoid DCs were depleted [24].

In the lungs of patients with chronic obstructive pulmonary disease (COPD), a lung disease caused by smoking, a clear accumulation of the Langerhans-type DC subset has been observed in the mucosa of small airways, suggesting a role for this myeloid DC subset in the pathogenesis of the disease [25, 26].

DCs are also found in malignant tumors. However, their activating function on the adaptive immune system is suppressed by cytokines, interleukins and

vascular endothelial growth factor, produced by the tumor. DCs are conditioned by the tumor to form suppressive T cells. By this mechanism, the tumor evades the IMMUNE SYSTEM [27].

DCs have also been implicated in the pathogenesis of several AUTOIMMUNE DISEASES. Under certain conditions, DCs can present self ANTIGENS and induce the expansion of autoreactive T cells. In addition, plasmacytoid DCs are proposed to be pathogenic in SYSTEMIC LUPUS ERYTHEMATOSUS, dermatomyositis and Sjögren's syndrome [28].

In the process of transplant rejection, DCs also play a crucial role. DCs from the grafted organ migrate into the recipient, where they stimulate alloreactive T cells, leading to graft rejection. In addition, DCs from the recipient can sample, process and present portions of the graft, eliciting the process of graft rejection [29, 30].

Pharmacological implications

Due to their strategic position in the IMMUNE SYSTEM, DCs are attractive TARGET cells for the development of new preventive and therapeutic strategies. However, since there are different DC phenotypes present (with different immunological functions), the modulation of a specific DC subset may be a suitable strategy, but this is difficult to achieve *in vivo*.

It is well known that glucocorticosteroids, used in medical practice as anti-inflammatory and immunosuppressive drugs, can reduce the number of myeloid and plasmacytoid DCs *in vivo* (see chapter C13). These effects are obviously not limited to DCs, as other cells are also dramatically influenced by the effects of glucocorticosteroids. Other immunosuppressive drugs such as rapamycin can also interfere with the maturation and function of DCs (see chapter C12).

In the quest for tolerogenic DCs, molecules with potential immunomodulatory functions have been tested for their abilities to modulate DCs *in vitro*. Examples include IL-10, 1α,25-dihydroxyvitamin D3, thymic stromal lymphopoietin and CTLA-4 IgG (used to block CD80 and CD86) [31] (see chapter C15). These immunomodulated DCs could be interesting in the future treatment of transplant patients at risk for graft rejection.

As key antigen-presenting cells, DCs are indispensible for successful vaccination. The ultimate goal of vaccination is the induction of an antigen-specific immune response with a long-lasting immunological memory to protect against subsequent disease (see chapter C1). Therefore, vaccine ANTIGENS need to be presented by DCs to activate the adaptive immune response. ADJUVANTS co-administered with the antigen directly or indirectly stimulate DCs to mature and present the ANTIGENS. Apart from promoting antigen multimerization and internalization, particulate adjuvants (including mineral salts such as alum) directly TARGET the INFLAMMASOME in the DC, inducing the production of several proinflammatory CYTOKINES [32].

In the past few years there has been much interest in developing DC vaccinations for the treatment of cancer and infections such as HIV. *Ex vivo*-generated DCs are loaded with the antigen of interest and administered to the patient to induce an adaptive immune response that overrules the tolerogenic environment and kills the cancer or virus-infected cells. These DC vaccinations have the potential advantage of strong activation of the cellular adaptive immune response, whereas conventional vaccination (antigen and adjuvant) mainly elicits a humoral immune response. However, DC vaccinations are currently in an experimental phase and, although results are encouraging, further research is warranted [33].

Summary

DCs are professional antigen-presenting cells of hematopoietic origin. They form a surveillance network in the skin, at the mucosal surfaces and in the internal organs. In these locations, DCs continuously sense for danger signals and sample ANTIGENS. DCs migrate towards the draining lymph nodes, process the sampled ANTIGENS and present them on MHC molecules to naïve T cells. Depending on the expression of COSTIMULATORY MOLECULES and the production of CYTOKINES mature DCs steer the T cell differentiation towards a Th1, Th2, Th 17 or regulatory T cell

response. Mature myeloid DCs are also important stimulators of effector T cell proliferation, whereas immature DCs and plasmacytoid DCs are more tolerogenic. In this way, DCs are indispensable to induce and modulate the adaptive immune response.

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Cytokines

Klaus Resch and Michael U. Martin

Introduction

In immune responses several cell types have to cooperate. Within the LYMPHOID ORGANS, such as lymph nodes, spleen or PEYER'S PATCHES, the cells of the IMMUNE SYSTEM interact in a dynamic fashion. At all times some cells, especially T LYMPHOCYTES, leave a lymphoid organ (such as a lymph node), circulate through the body and enter another lymphoid organ in search of a fitting antigen. As a consequence, the communication between the cells of the IMMUNE SYSTEM – LYMPHOCYTES, monocytic cells and GRANULOCYTES – depends on secreted diffusible mediators, the most important of which are the CYTOKINES.

There is no unambiguous definition for these molecules, with the exception that they are all proteins and are mostly glycosylated. The majority of CYTOKINES are not synthesized exclusively by the cells of the IMMUNE SYSTEM, but also by several other cell types. In turn, CYTOKINES too may have effects on many different cells. Therefore, they cannot be clearly distinguished from the general GROWTH FACTORS, most of which also affect cells of the IMMUNE SYSTEM, although their primary function, e.g., that of the nerve growth factor NGF, involves other organs. For practical purposes CYTOKINES can be defined as protein mediators that are: (1) primarily synthesized by cells of the IMMUNE SYSTEM; (2) regulate predominantly differentiation and activation of immune cells; and (3) are responsible for various effector functions of LEUKOCYTES, including INFLAMMATION as an important example.

The field of cytokine biology as it stands today has three major roots. The first originates in immunology in the 1960s, when it was demonstrated that supernatants of stimulated immune cells could regulate function and growth of LEUKOCYTES. Partially purified factors were published under function-related names such as lymphocyte activation factor (LAF) or T cell growth factor (TCGF), which according to their major producer cells were grouped as monokines or lymphokines. In the seventies, the term CYTOKINES was then gradually adapted (upon suggestion by S. Cohen, see [1]) as a more general denomination. As it became apparent that identical molecules had been described under different names, the term INTERLEUKINS was proposed at the 2nd International Lymphokine Workshop held in 1979 "as a system of nomenclature … based on the ability to act as communication signals between different populations of LEUKOCYTES", and concomitantly the names INTERLEUKIN-1 (IL-1) for LAF and INTERLEUKIN-2 (IL-2) for TCGF were introduced (see [1]).

The second source of cytokine research stems from virology and classifies the INTERFERONS, originally described in 1957 by A. Isaacs and J. Lindemann (see [1]), as factors produced by virus-infected cells capable of conferring resistance to infection with homologous or heterologous viruses. Although this activity still defines the groups of INTERFERONS, it soon became apparent that they also regulate immune responses, which make them a subgroup of CYTOKINES. Regulation of immune and inflammatory reactions is the predominant function of one member, IFN-γ, also called immune interferon, which is structurally different from the large family of IFN-α/β INTERFERONS.

COLONY-STIMULATING FACTORS (CSFs), which form the third root of CYTOKINES were first described in 1966 by D. Metcalf, reflecting the observation that they promote the formation of granulocyte or monocyte colonies in semi-solid medium. CSFs predominantly function as proteins that induce growth and differentiation of HEMATOPOIETIC STEM CELLS, but can also – as was recognized later – activate fully differentiated cells. A more detailed description of the history of CYTOKINES can be found in the introductory chapter by J. Vilček in *The Cytokine Handbook* [1].

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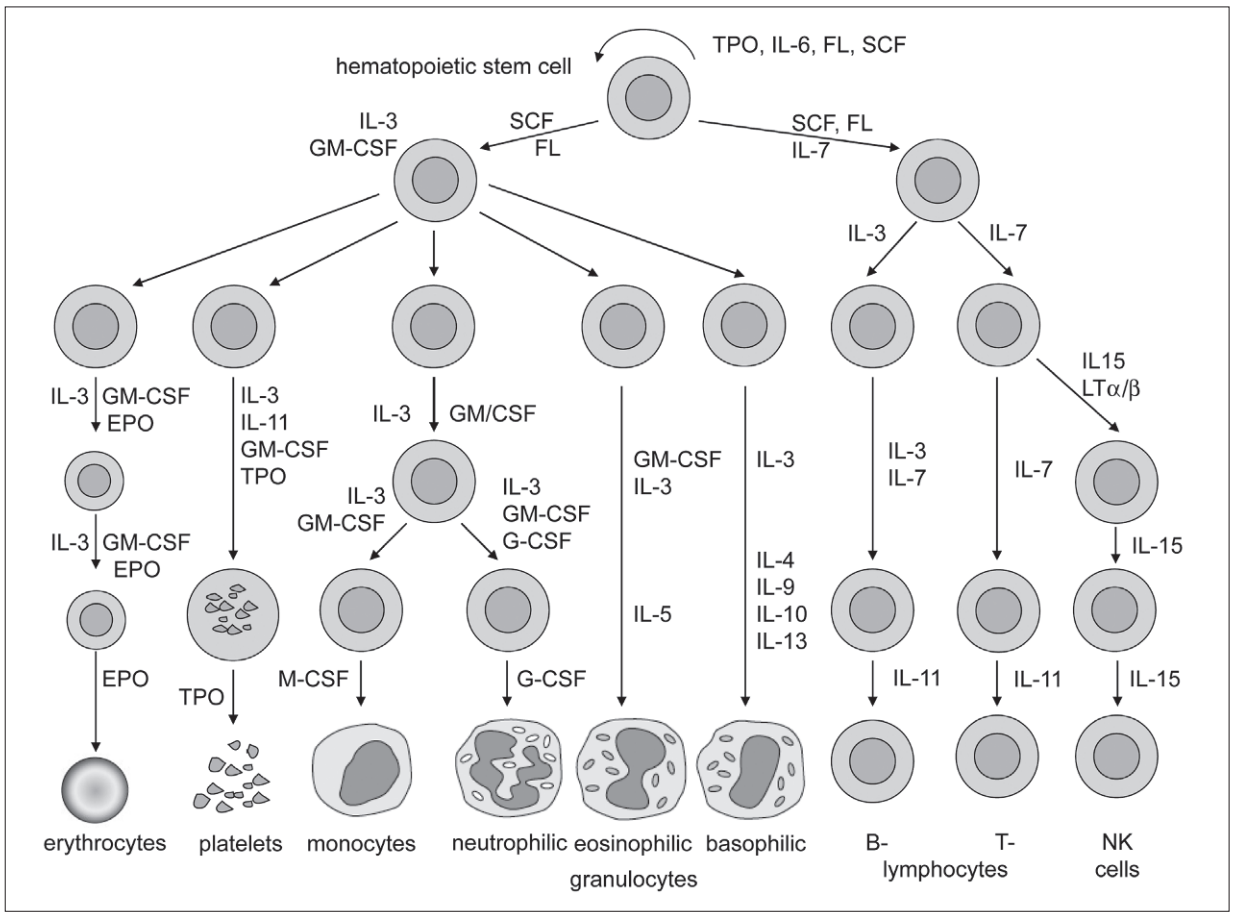

Figure 1. Cytokines involved in the differentiation of cells of the immune system
CSF, colony-stimulating factor; EPO, erythropoietin; FL, Flt ligand; G, granulocyte; IL, interleukin; LT, lymphotoxin; M, monocyte; SCF, stem cell factor; TPO, thrombopoietin

All CYTOKINES exert their biological functions by binding to high-AFFINITY plasma membrane RECEPTORS. The identity of the known CYTOKINES and their respective RECEPTORS has been elucidated by molecular cloning [1–4].

Differentiation factors

The mature cells of the IMMUNE SYSTEM have a finite life span. Their physiological half-life ranges from several hours (neutrophilic GRANULOCYTES) to months (MONOCYTES and most LYMPHOCYTES). Only some lymphocyte subsets survive longer, memory T helper (Th) LYMPHOCYTES for many years. Therefore, the cells must be continuously renewed from a stem cell pool, which in adult human beings is located in the BONE MARROW. How the reservoir of STEM CELLS is kept constant by self renewal is not entirely clear. Besides a direct influence of the stromal (local tissue) microenvironment, a number of CYTOKINES has been implicated, including thrombopoetin (TPO), Flt LIGAND (FL), stem cell factor (SCF) and INTERLEUKIN

Table 1. Myeloid differentiation factors, erythropoietin and thrombopoietin

	Molecular mass (kDa)	Predominant producer cells	Major functions
Stem cell factor	36	Bone marrow stromal cells	Self renewal and differentiation of stem cells
Flt3 ligand (FL)	25 (m)[1] 16 (s)[2]	Bone marrow stromal cells	Self renewal and differentiation of stem cells
IL-3	14–28	T lymphocytes	Differentiation and propagation of myeloid progenitor cells
Granulocyte-monocyte CSF	14–35	T lymphocytes, monocytes, endothelial cells, fibroblasts	Differentiation and propagation of myeloid progenitor cells
Granulocyte CSF	18–22	Monocytes	Propagation and maturation of granulocytes
Monocyte CSF	35–45 18–26	Endothelial cells Fibroblasts, monocytes	Propagation and maturation of monocytes
Erythropoietin	30–32	Peritubular renal capillary cells	Maturation of erythrocytes
Thrombopoietin	31	Liver, kidney	Maturation of platelets, self renewal of stem cells

[1] membrane bound, [2] soluble
CSF, colony stimulating factor

(IL)-6, in some instances also IL-1. The HEMATOPOIETIC STEM CELLS, of which low numbers are also present in blood – carrying the surface marker CD34 – give rise to lymphocytic or myelomonocytic cells, as well as to platelets and ERYTHROCYTES (Fig. 1) (see also chapter A1).

Differentiation of cells of the myelomonocytic cell lineage

The major differentiation factors of the myelomonocytic cell lineage are well known (Tab. 1). Several of them are named COLONY-STIMULATING FACTORS (CSFs) according to the observation, leading to their discovery, that they can stimulate outgrowth of colonies from BONE MARROW cell cultures. Some of these factors, such as stem cell factor (SCF) and multi-CSF (synonymous with IL-3), regulate early differentiation steps. Others, such as granulocyte/monocyte (GM)-CSF control intermediate steps or selectively induce end differentiation into mature (neutrophilic) GRANULOCYTES (G-CSF) or MONOCYTES (M-CSF). Similarly, erythropoietin, synthesized in the kidney, promotes generation of ERYTHROCYTES and thrombopoietin, which is synthesized in the liver and spleen, promoting formation of platelets. Table 1 summarizes the predominant physiological properties of these proteins [5].

Pharmacological implications

As gene technology has facilitated the production of sufficient amounts, CSFs are now exploited as drugs (Tab. 2) [5,6]. Thus, erythropoietin has become established as the drug of choice for the treatment of severe anemias during terminal renal diseases or due to cytostatic therapy [7]. Thrombopoetin has been applied successfully in clinical trials for the treatment of thrombocytopenias [8]. FILGRASTIM (human RECOMBINANT G-CSF with an additional meth-

Table 2. Possible Indications for cytokines
I. Reconstitution of a compromised immune system (physiological effects)

	Target cell	Indication
Epo*	Erythroid progenitor cells	Anemia
Tpo	Megakaryocytic progenitor cells	Thrombocytopenia
G-CSF	Myeloid progenitor cells	Granulocytopenia
M-CSF	Monocytic progenitor cells	Monocytopenia
GM-CSF	Myelomonocytic progenitor cells	Leukopenia
IL-3	Myelomonocytic progenitor cells	Leukopenia
IL-2	Lymphocytic progenitor cells	Lymphopenia
IL-7	Lymphocytic progenitor cells	Lymphopenia
IL-11	Lymphocytic progenitor cells	Lymphopenia
	Megakaryocytic progenitor cells	Thrombocytopenia
IL-6	Hematopoietic stem cells	Stem cell deficiency expansion of stem cells *in vitro*

Epo, erythropoietin; Tpo, thrombopoietin; CSF, colony stimulating factor; G, granulocyte; M, macrophage, IL, interleukin
**bold letters indicate those that have been approved; others in clinical evaluation*

ionine, generated from bacteria) was the first CSF approved for the treatment of granulocytopenias. Similarly to LENOGRASTIM (human RECOMBINANT G-CSF from eukaryotic cells), it promptly and selectively increases up to 100-fold the number of functionally active NEUTROPHILS, for instance in patients with CYTOTOXIC DRUG-induced neutropenias. To increase their half-lives *in vivo*, CSFs were covalently linked to polyethylene glycol (PEGYLATION). Treatment with G-CSF markedly reduced the incidence and severity of infections leading to hospital admissions in patients who had received chemotherapy because of malignant tumors. In tumor patients, however, the therapy has not led to an increase in life expectancy. GM-CSF (MOLGRAMOSTIM) has been approved for similar indications. All other CSFs have been evaluated in clinical trials, and some are approved in countries outside Europe.

Therapeutically administered CSFs are intended to substitute for the loss of a patient's own differentiation factors. Nevertheless, these "BIOLOGICALS", like all drugs, can cause side effects. For the CSFs these include bone and muscle pain, dysuria, sometimes elevation of liver enzymes and uric acid, and rarely, a drop in blood pressure, eosinophilia, or allergic reactions [6].

Lymphocyte differentiation

Differentiation of T and B LYMPHOCYTES from STEM CELLS proceeds in a more complex way. The central process consists of the generation of the huge (about 10^8 in human beings) diversity of T and B cell antigen-specific RECEPTORS. The central element involved is the free combination of a finite number of gene elements at the level of DNA during the differentiation of the cell lineages. For this somatic gene rearrangement, IL-7 appears to be indispensable, in B as well as in T LYMPHOCYTE development. Addition-

ally, less well-characterized CYTOKINES are involved in the maturation of T LYMPHOCYTES in the thymus or of B LYMPHOCYTES in the BONE MARROW, including for instance IL-11 [9]. Both IL-7 and -11 have been tested clinically in patients with lymphopenias.

Activation and growth factors of lymphocytes

To carry out their specific functions immune cells have to be activated. The activation of naïve B LYMPHOCYTES is initiated by binding of the specific ANTIGENS to their antigen RECEPTORS, plasma membrane-bound IgM and IgD. This can lead to secretion of small amounts of IgM. The expansion of the antigen-reactive B cells and, concomitantly, effective immunoglobulin synthesis requires additional stimuli provided by IL-4, IL-6, IL-10 and IL-13 [10] (see chapter A3). These INTERLEUKINS are synthesized and secreted by a subpopulation of $CD4^+$ T helper LYMPHOCYTES, the Th2 cells. They not only promote the maturation of Ig-producing B LYMPHOCYTES to the fully secretory plasma cell, but they also control switching to the synthesis of other immunoglobulin ISOTYPES. These include IgG, which is important for an effective antibacterial defense, or IgA, which is secreted through mucosal linings and thus exerts an early line of defense before entry of bacteria. For the synthesis of IgE, which is responsible for defense against multicellular parasites as well as type I allergic reactions, IL-4 or IL-13 are of pivotal importance (Fig. 2, page 66) (see chapters A8 and A9).

Activation of T LYMPHOCYTES also requires participation of CYTOKINES [11]. To ensure an effective cellular immune defense, antigen-reactive T LYMPHOCYTES must proliferate and thereby expand clonally; from a single lymphocyte up to 10^7 descendants may originate in this way (see chapter A2). The predominant T LYMPHOCYTE growth factor is IL-2, which, if absent, may be substituted by IL-15. IL-2 is formed by the second $CD4^+$ T helper subpopulation, the Th1 cells, and acts on CYTOTOXIC ($CD8^+$) T LYMPHOCYTES as well as on T helper LYMPHOCYTES themselves [12]. CYTOTOXIC T LYMPHOCYTES can kill TARGET cells presenting antigenic peptides, most importantly virus-infected cells. Activated Th1 cells, by secreting IFN-γ [and other mediators such as TUMOR NECROSIS FACTOR (TNF) β], initiate inflammatory reactions (see below). It should be noted that, by secreting IL-3 and GM-CSF, these T cells also stimulate the formation of monocytic and myeloid cells in the BONE MARROW, which are required for inflammatory reactions. The INTERLEUKINS that have been defined so far at a molecular level are summarized in Table 3.

INTERLEUKINS mediate interactions between the different LEUKOCYTES. More than 30 members have been ascribed so far to this rather HETEROGENEOUS group of proteins. Interestingly, some more "novel" INTERLEUKINS exhibit similar properties to those detected earlier. For example, IL-13 exhibits similar properties to IL-4 as it binds to the IL-4 RECEPTOR as well as to its own RECEPTOR. IL-21 is related to IL-2 and to IL-15 [12]. Based on structural homologies, some INTERLEUKINS have been grouped into families; IL-2, IL-15 and IL-21 receptors form the IL-2 family; and IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29 and IL-10 constitute the IL-10 family [13]. CYTOKINES, besides binding to their unique RECEPTOR chains, may share a common RECEPTOR chain, e.g., IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors share the common RECEPTOR γ-chain (γ_c). IL-6, IL-11, IL-21, oncostatin M, ciliary neurotropic factor and leukemia-inhibiting factor use gp130 together with individual RECEPTOR chains [4]. This shows that for functionally important INTERLEUKINS, SURROGATE molecules exist, which, at least partially, can take over the function of the former when it is synthesized in insufficient amounts. The redundancy clearly indicates the importance of a functioning IMMUNE SYSTEM for the survival of a species. Other factors with overlapping functional properties are likely to be responsible for the fine tuning of immune responses [14–23].

Pharmacological implications

Although many of the CYTOKINES implicated in the activation of T or B LYMPHOCYTES have been considered for the treatment of predominantly malignant tumors, only IL-2 (aldesleukin) so far has been approved for a very limited indication, namely metastasizing renal carcinoma. In combination with other

Table 3. Interleukins

Cytokine	Molecular mass (kDa)	Predominant producer cells	Major functions
IL-1α IL-1β	17	Monocytes	Activation of T lymphocytes and inflammatory cells
IL-1 ra	17	Macrophages	Complete IL-1 receptor antagonist
IL-2	15	T lymphocytes	Proliferation of T lymphocytes, promonocytes, NK cells
IL-3	14-28	T Lymphocytes	Differentiation and propagation of early myeloid progenitor cells
IL-4	15-20	T Lymphocytes	Differentiation and proliferation of Th2 cells and B lymphocytes, inhibition of macrophage activation
IL-5	45-60	T Lymphocytes	Maturation of eosinophils
IL-6	26	T lymphocytes, many other cells	Activation of B and T lymphocytes and other cells, stem cell expansion
IL-7	25	Stromal cells	Maturation of T and B lymphocytes
IL-8	10	Monocytes/ macrophages	Chemotaxis and activation of granulocytes, chemotaxis of T lymphocytes
IL-9	37-40	T lymphocytes	Propagation of mast cells, megakaryocytes
IL-10	17-21	T lymphocytes	Inhibition of cellular immune and inflammatory reactions, propagation of mast cells,
IL-11	23	Stromal cells	Maturation of lymphocytes, proliferation of myeloid and megakaryocytic progenitor cells
IL-12	p35/p40 dimer	Monocytes/ macrophages, dendritic cells	Differentiation and activation of Th1 lymphocytes, NK cells
IL-13	17	T lymphocytes	Activation and proliferation of B lymphocytes, inhibition of cellular immune reactions
IL-14	60	T lymphocytes	Activation of B lymphocytes,
IL-15	14-15	Epithelial cells	Activation and proliferation of T lymphocytes, NK cells
L-16	14	T lymphocytes	Chemotaxis of T lymphocytes, macrophages
IL-17	35 homodimer	T lymphocytes	Induction of pro-inflammatory cytokines
IL-18	24	Monocytes/ macrophages, dendritic cells	Differentiation and activation of Th1 lymphocytes
IL-19	18	Monocytes	Differentiation of Th2 lymphocytes
IL-20	18	Monocytes, keratinocytes	Skin and kidney inflammation, down regulation of T cell responses
IL-21	14	T helper lymphocytes, esp. Th2, Th17 cells	Regulation of B cell differentiation and activation, differentiation of NK cells

Table 3. (continued)

Cytokine	Molecular mass (kDa)	Predominant producer cells	Major functions
IL 22	16.5 (monomer)	Th17 lymphocytes, mucosal NK cells	Augmentation of innate immune reactions in skin and mucosa
IL-23	60 (dimer of p19 and IL-12 p40)	Monocytes, dendritic cells	Th17 differentiation, activation of monocytes
IL-24	33	Monocytes,Th2 cells	Induction of inflammation in skin, lung
IL-25	18	Mast cells, eosinophils, Th2 cells	Induction of Th2 cytokines (IL-4,-5,-9,-13)
IL-26	17,7 (monomer)	T lymphocytes, NK cells	Local immune mechanisms in skin or lung, induction of IL-8, IL-10
IL-27	60 (dimer of p34 +p28)	Dendritic cells, placentary trophoblasts	Regulation of differentiation of Th1 cells, suppression of Th17 development
IL-28 A and B	24 and 22	Lymphocytes, dendritic cells, virus-infected cells	Induction of type I interferons, development of tolerogenic DC and Treg
IL-29	26-35	Like IL-28	Like IL-28
IL-30			Reported as p28 chain of IL-27
IL-31	24	Th2 cells	Hematopoiesis , modulation of cell proliferation of many cell types
IL-32	25	T lymphocytes, NK cells	Differentiation and activation of monocytes
IL-33	32	Endothelial cells, smooth muscle cells, keratinocytes	Increase in Th2 cytokine release, activation of mast cells, basophils and eosinohils
IL-34	39 (monomer)	Many cells	Proliferation of monocytes and monocytic progenitors,
IL-35	70 (dimer of IL-12 p35 + IL-27b)	Treg, dendritic cells (?), placentary trophoblasts	Control of T effector cells, immune modulator at feto-maternal barrier in placenta

IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29, together with IL-10, constitute the IL-10 family
IL-21, together with IL-2 and IL-15, constitute the IL-2 family
IL-23 is related to IL-12 (dimer of the IL-12p 40 chain plus separate p19 chain)
IL-31 belongs to the IL-6 family, together with IL-11, oncostatin (OSM), ciliary neurotropic factor (CNF), and leukemia-inhibitory factor (LIF)
IL-33, together with IL-1α, IL-1β, IL-1ra and IL-12, belongs to the IL-1-family

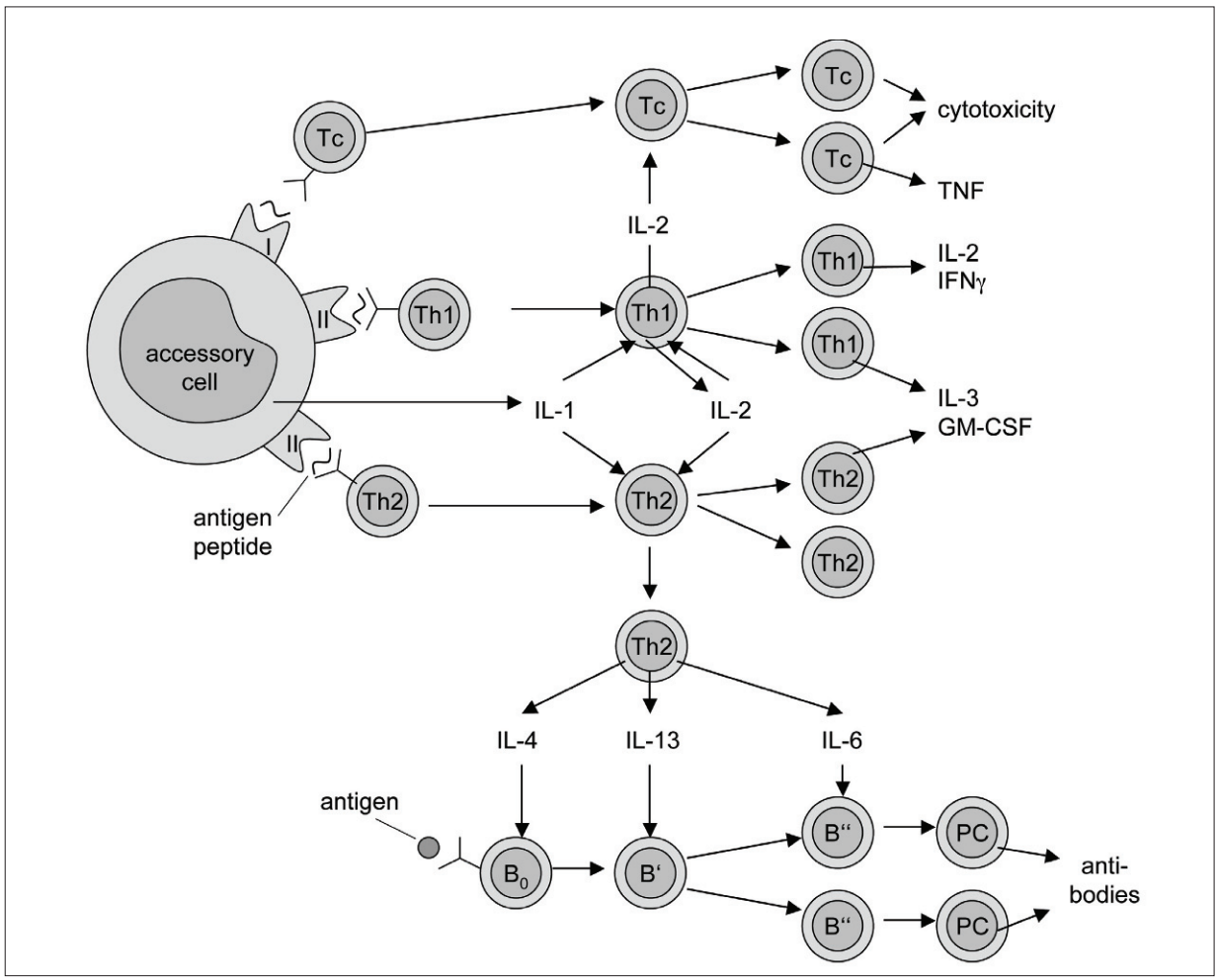

Figure 2. Activation of T and B lymphocytes

B, B lymphocyte; B_0, virgin B lymphocyte; B', B'', activated B lymphocytes; GM CSF, granulocyte/monocyte colony-stimulating factor; IFN, interferon; IL, interleukin; PC, plasma cell; Th, T helper lymphocyte; Te, T effector lymphocyte; TNF, tumor necrosis factor; I, MHC class I molecule; II, MHC class II molecule

activation factors, IL-2 is used *in vitro* to expand lymphokine-activated killer (LAK) cells, which emerge as a therapeutic regimen for some tumors (see chapter C6). In contrast, INTERLEUKINS have developed into a major TARGET for suppressing immune and inflammatory reactions involved in autoimmune or chronic inflammatory diseases, or transplant rejection (see below)

Mediators of inflammation

Mechanisms and pathophysiology

In response to their specific antigen, B LYMPHOCYTES secrete ANTIBODIES and antigen-reactive T LYMPHOCYTES expand clonally and become EFFECTOR CELLS. ANTIBODIES may immediately deal with ANTIGENS, for

example by neutralizing poisons. Similarly, CYTOTOXIC T LYMPHOCYTES can kill virus-infected cells directly. The major proportion of the defense, however, is generally exerted by inflammatory mechanisms [24]. To a large extent, these consist of the infiltration and activation of cells of the INNATE IMMUNE SYSTEM, LEUKOCYTES such as mononuclear PHAGOCYTES and various GRANULOCYTES [25, 26]. ANTIBODIES direct the LEUKOCYTES specifically to the antigen, and together with the mediators formed by Th1 cells, they greatly enhance the inflammatory response [27].

As a typical example, the activation of MONOCYTES/MACROPHAGES is depicted in Figure 3. Besides GM-CSF, which not only induces the differentiation of MONOCYTES and GRANULOCYTES, but also activates mature cells, IFN-γ and TNF-β constitute the most important macrophage-activating factors.

IFN-γ, generated by Th1 LYMPHOCYTES, is a member of the protein family of INTERFERONS; IFN-α is formed mainly by a subpopulation of DENDRITIC CELLS (DC2) and IFN-β by fibroblasts (Tab. 4) [28, 29].

In addition to their antiviral activity, all INTERFERONS can activate cells, most notably the nonspecific cells of the INNATE IMMUNE SYSTEM, i.e., MACROPHAGES and GRANULOCYTES, to a varying extent.

TNF-β is related to TNF-α, which is synthesized mainly by MACROPHAGES. As suggested by their name, both possess antitumor activities [30]. TNF-α and -β were recently assigned to a large super family of distantly related proteins, the TNF superfamily ligands (TNFSFL) with more than 20 members. Most of them are type 2 transmembrane proteins (the extracellular section of which can be released in a soluble form) and only TNFSFL1 (TNF-β) and TNFSFL2 (TNF-α) represent true CYTOKINES and are listed in Table 5.

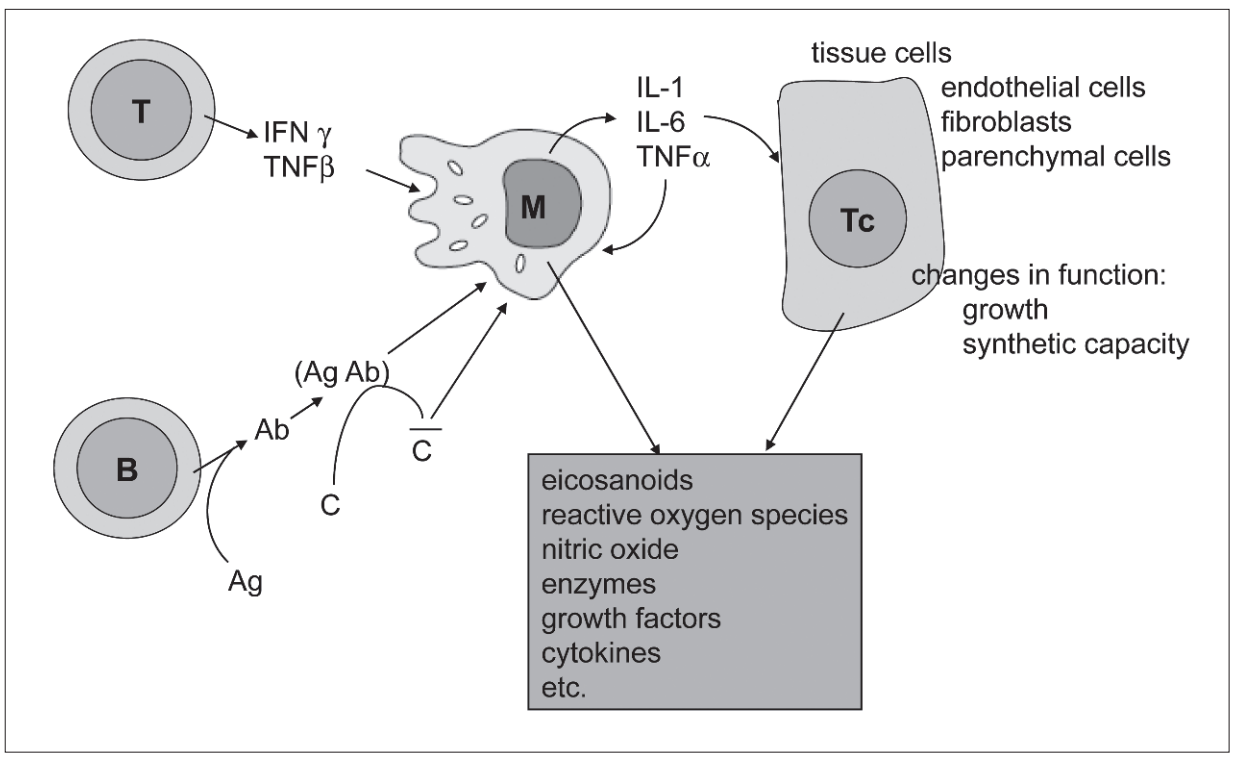

FIGURE 3. IMMUNE REACTIONS AND INFLAMMATION

Ag, antigen; Ab, antibody; B, B lymphocyte; C, complement; C̄, activated complement components; IFN, interferon; IL, interleukin; M, macrophage; TC, tissue cells; TNF, tumor necrosis factor

Table 4. Interferons

Cytokine	Molecular mass (kDa)	Predominant producer cells	Major functions
IFN-α ~15 proteins IFN-α1 IFN-α2 … etc.	19–26	Dendritic cells	Induction of antiviral activity Inhibition of tumor cell growth Activation of cells
IFN-β	23	Fibroblasts	Induction of antiviral activity
IFN-γ	17–25	T lymphocytes	Induction of antiviral activity Activation of macrophages Immunoregulation

Table 5. Tumor necrosis factor superfamily, TNFSF ligands (selection)

Cytokine		Molecular mass (monomers)[1] (kDa)	Predominant producer cells	Major functions
Commonly used name	TNFSL			
TNF-α	2	17 (s)[2]	Monocytes Many other cells	Defense against pathogens Inflammation Induction of apoptosis
TNF-β (=LTα)	1	25 (m)[3] 17(s)	T lymphocytes	Cachexia, shock
LTβ	3	33 (m)	T lymphocytes	Development, homeostasis and structure of lymphoid organs, virus defense
CD40L	5	29 (m)	T lymphocytes	B lymphocyte activation
FasL (CD95L) TRAIL	6 10	31 (m) 30 (m)	T lymphocytes T lymphocytes	Induction of apoptosis

CD, cluster of differentiation; Fas, death receptor; L, ligand; LT, lymphotoxin; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand
[1] Generally forming multimers such as homotrimers
[2] As soluble protein
[3] As membrane bound protein

Importantly, IL-1, TNF and IL-6, effectively activate many tissue cells, including endothelial and parenchymal cells to secrete the effector molecules of INFLAMMATION [30–32]. In this way, these tissue cells are recruited to contribute to an inflammatory reaction. In addition, these CYTOKINES also enhance the activities of the mononuclear PHAGOCYTES and other LEUKOCYTES in an autocrine and paracrine

way, thereby amplifying the inflammatory reaction by up-regulating the synthesis and release of other pro-INFLAMMATORY CYTOKINES such as IL-17 [14]. These dual properties give these "master CYTOKINES" a central role as mediators of INFLAMMATION. The boosting of inflammatory defense mechanisms against infectious agents and tumors constitutes the basis for attempts to positively modulate infectious diseases or malignant tumors by the administration of CYTOKINES (Tab. 6) (see chapters A8, C6 and C7).

Pharmacological relevance

Approved indications for NATURAL IFN-β include severe virus infections such as recurring *varicella zoster* infections or *herpes simplex* infections of the eye. RECOMBINANT IFN-β (IFN-β1a or –1b) is indicated in some forms of MULTIPLE SCLEROSIS [33].

IFN-α (IFN-α1a, and IFN-α2b) is indicated in chronic hepatitis B and hepatitis C (see chapter C7), as well as in the treatment of some malignant tumors, including hairy cell leukemia or chronic myeloid leukemia [34]. It is effective against some other tumors such as non-Hodgkin lymphoma, cutaneous T cell leukemia, malignant melanoma, hypernephroma or bladder carcinoma, but it is ineffective against the majority of carcinomas. Antitumor effects of IFN-β and IFN-γ in humans are uncertain. Conjugates of IFN-α2a or -2b with polyethylene glycol (PEG-IFN-α2a or -2b) have been introduced for the treatment of hepatitis B and C. These conjugates are slowly taken up from a subcutaneous depot and also excreted slowly, thus providing sustained blood levels.

Clinical side effects of INTERFERONS include the common "flu-like" syndrome (fever, fatigue, shivering, muscle and joint pain), paresthesias, disturbances of the CENTRAL NERVOUS SYSTEM, depression, gastrointestinal disturbances, cardiac symptoms, granulocytopenia, thrombopenia or anemia [29].

The CYTOKINES TNF-α, IL-1, IL-2, IL-12 and IL-18 have been the subjects of clinical trials for treating malignant tumors (see chapter A10 and C6). Despite some positive results – such as with IL-2 in metastasizing renal cell carcinoma, the indication for which it has been approved, or melanoma [35] – the overall outcome so far has been rather disappointing. The major reason for this appears to be limitation due to the severe toxicity that arises when these CYTOKINES are administered systemically. This has prompted attempts to increase CYTOKINE concentration locally in the tumor by perfusing it, for instance, with TNF. As experiments in animal models have been encouraging, this strategy is being followed in clinical trials with limb tumors or hepatocarcinomas [36]. Another strategy that is being followed is to transfect either tumor cells or tumor-infiltrating cells so that they con-

TABLE 6. POSSIBLE INDICATIONS FOR CYTOKINES
II. Activation of normal cells of the immune systems (pharmacodynamic actions)

Cytokine	Target cells	Indication
IFN-α*	Virus-infected cells, tumor cells	Viral infections, malignant tumors
IFN-β	Virus-infected cells	Viral infections
TNF-α, β	Monocytes/macrophages, tumor cells	Malignant tumors
IL-2	T lymphocytes, monocytes, NK cells	Malignant tumors, viral infections (AIDS)
IL-6	Hematopoietic stem cells	Expansion of stem cells

IFN: interferon; IL: interleukin; TNF: tumor necrosis factor
**those in bold letters have been approved, others are in clinical evaluation*

stitutively secrete high amounts of a CYTOKINE such as IL-2 or TNF. After reinfusion and subsequent redistribution to sites of the tumor, a high local concentration of the CYTOKINES in the tumor may be achieved, which could support sufficient antitumor activity but be accompanied by tolerable systemic effects [37]. A number of clinical trials of this GENE THERAPY have been initiated. It should be added that the greatest utility of CYTOKINES in the treatment of cancer so far has been for the management of cancer or cancer therapy-associated side effects such as a decrease in white blood cells, anemia or thrombocytopenia. This includes G-CSF, erythropoietin, thrombopoietin IL-3, IL-7 or IL-11 (see above) [38].

Modern molecular biological methods allow the composition of proteins to be changed to yield new or altered functional properties. Thus, a desired effect could be enforced, and simultaneously an unwanted side effect reduced. This concept has been applied to CYTOKINES, and many new functional proteins have been created ("designer CYTOKINES", "super"-CYTOKINES", "MUTEINS"). One example is a fusion protein of IL-6 and the extracellular domain of its RECEPTOR ("hyper IL-6"), which (due to the fact that this complex can also activate cells containing only gp130, the second, non-LIGAND binding chain of the IL-6 RECEPTOR) is more than 100-fold as potent as IL-6. This construct is now used to expand HEMATOPOIETIC STEM CELLS *in vitro* [39].

Infection with HIV leads to progressive and preferential loss of $CD4^+$ T helper cells. Death results from OPPORTUNISTIC INFECTIONS or, less often, malignant tumors. In combination with effective antiviral treatment strategies (i.e., at least triple combinations of drugs such as zidovudine, didanosine and atazanovir), IL-2 can ameliorate immunodeficiency (see chapter C7).

Regulatory factors of immune reactions

The Th1/Th2 paradigm

While it is desirable in infectious diseases or malignant tumors to augment immune and subsequent inflammatory reactions, in many other situations, such as autoimmune, chronic inflammatory or allergic diseases, they are pathogenic. Under physiological conditions, immune reactions are tightly controlled, and immunological diseases may therefore be regarded as failures of immunoregulation. As a central control mechanism, the balance between the activity of Th1 and Th2 cells has emerged (Fig. 4, see also Fig. 2) [40, 41].

Th1 cells secrete the CYTOKINES IL-2 and IFN-γ, which, as described above, promote cell-mediated defense and inflammatory responses. Th2 cells, on the other hand, synthesize those CYTOKINES that predominantly regulate the activation of B LYMPHOCYTES, i.e., IL-4, IL-6 and IL-13. Both Th subpopulations develop from common $CD4^+$ precursors, Th0. IFN-γ and IL-12 together with IL-18 (synthesized mainly by activated MACROPHAGES) promote differentiation into Th1 cells, whereas IL-4 is responsible for Th2 differentiation. Simultaneously, the subpopulation-specific CYTOKINES block development of the opposite Th subpopulation. This implies that if one subpopulation gains a developmental advantage, this is reinforced while the corresponding subpopulation is suppressed.

Since no Th1- or Th2-specific ANTIGENS or EPITOPES have been detected, the initial channeling of preferential differentiation into a specific Th subpopulation – and thereby the type of the ensuing immune reaction – must be directed by other cells. For Th1 cells, this is a function of MONOCYTES/MACROPHAGES and especially DENDRITIC CELLS, which, following interaction with bacteria or their components (e.g., LIPOPOLYSACCHARIDE), release IL-12 and IL-18. For Th2 cells, MAST CELLS play a similar role, since they contain vast amounts of IL-4, which can be released in direct contact with a number of known ALLERGENS or parasitic ANTIGENS.

More recently, it has become clear that further Th subpopulations exist that exert very specific functions. An example are the Th17 cells, which, besides minor amounts of CYTOKINES of the IL-10 family (see above), predominantly synthesize IL-17, which is implicated mostly in autoimmune reactions [14, 41, 42].

As a central new subpopulation, the regulatory T cells (Treg) have emerged, the major function of which is the suppression of immune responses (Fig. 5).

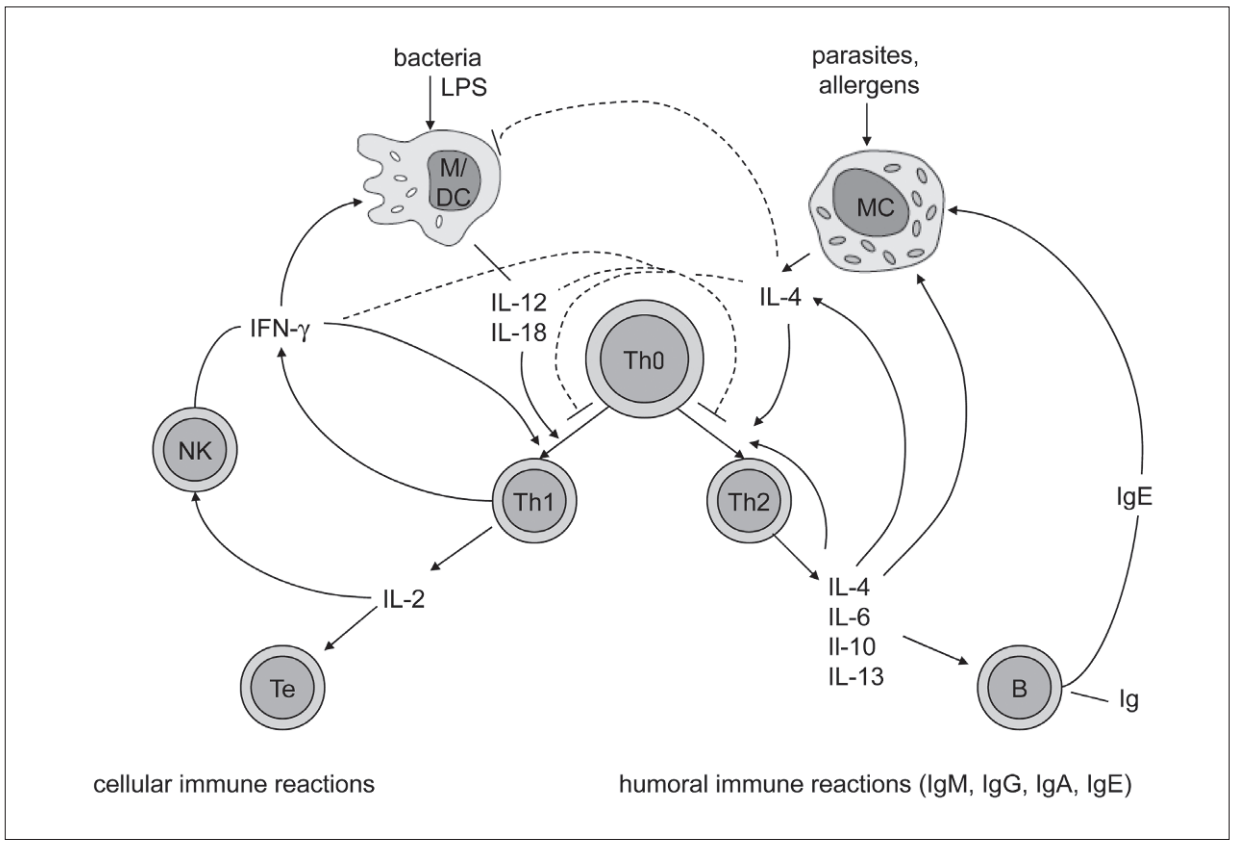

Figure 4. Regulation of immune responses

B/M, basophilic granulocytes/mast cells; B, B lymphocytes; IFN, interferon; Ig, immunoglobulin; IL, interleukin; M, macrophage; NK, natural killer cell; Th, T helper cell; Th0, T helper cell precursor; Tc, cytotoxic T cell

At present two different types of Tregs are known, which are distinguished by their origin and function. Natural Treg (nTreg) develop in the thymus and predominantly recognize autoantigens. They suppress autoimmune T lymphocyte reactions mainly by cell-cell contact. Their major role thus appears to maintain self tolerance. Induced Treg (iTreg) develop from T helper precursors (Th0) in the periphery, which involves transforming growth factor-β (TGF-β). iTreg interact with tissue antigens, but can also be activated by foreign antigens. The latter property enables iTreg to generally dampen immune reactions. They can thus hinder excessively strong, self-destructive inflammatory reactions from developing or persisting, for instance in acute infections. iTreg suppress effector cells predominantly by secreting suppressive cytokines such as IL-10 or TGF-β [43, 44].

Pharmacological implications

It is obvious from these data that IL-4, IL-13 and especially IL-10 should exert anti-inflammatory effects in non-allergic situations. Indeed, this is supported by many *in vitro* experiments and, more relevant, experimental animal models. In these studies, it was found that not only development and activation of Th1 cells was inhibited, but also the activation of macrophages, the predominant "chronic" inflammatory cells. All these Th2 cytokines, therefore, are presently

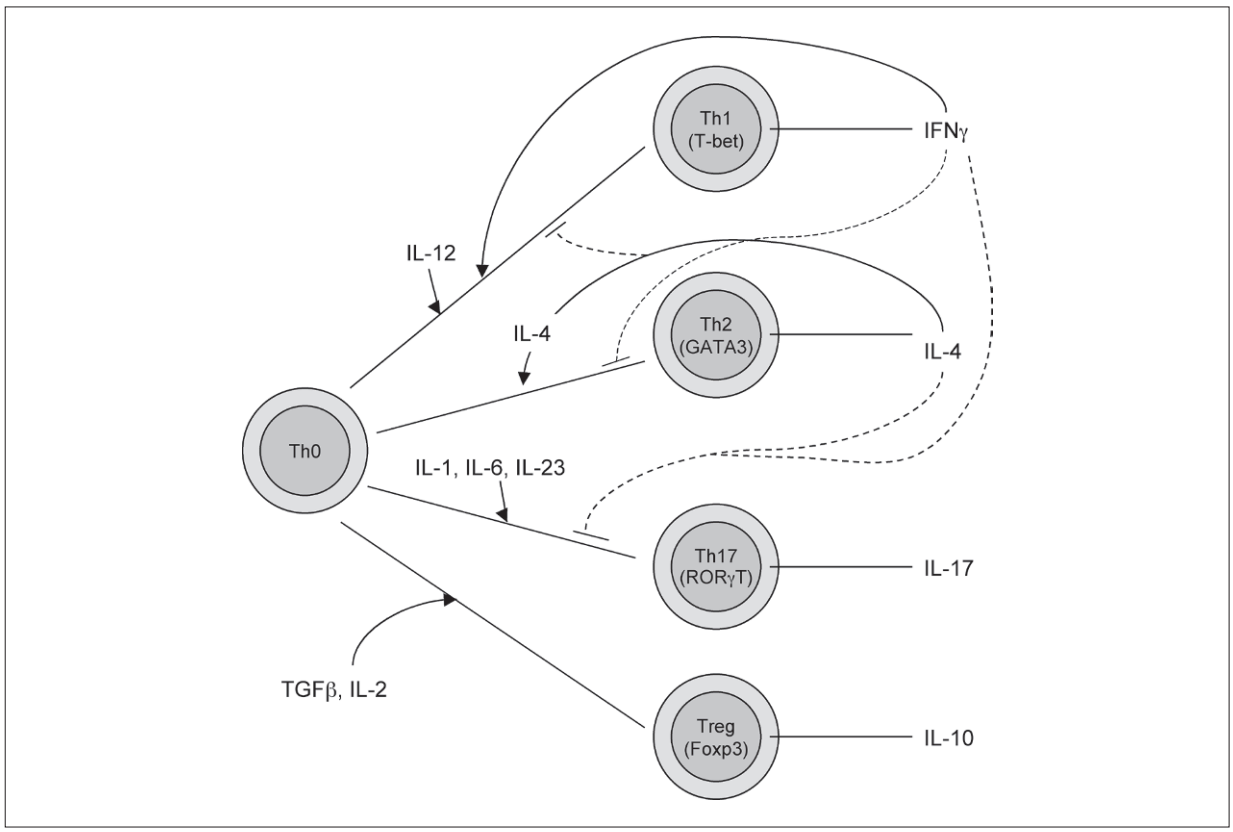

Figure 5. Development of T helper subpopulations
IL, interleukin; IFN, interferon; Th0, T helper cell precursor; TGF, transforming growth factor; Tbet, GATA3, RORγ-T, Foxp3: transcription factors

undergoing clinical evaluation in several chronic inflammatory diseases, including RHEUMATOID ARTHRITIS (see chapter C15).

Chemokines

Pathophysiology

Inflammatory – as well as allergic – diseases usually are confined to certain organs. This implies that all cells of the IMMUNE SYSTEM that participate in the underlying pathomechanisms must emigrate from the blood stream and invade into the perspective tissue. On the other hand, antigenic material from infective agents penetrating into the body must be taken up by antigen-presenting DENDRITIC CELLS, and in this form carried to the adjacent LYMPHOID ORGANS, such as the regional lymph nodes, to initiate an effective immune response. The very complex migration of LEUKOCYTES, which proceeds in several subsequent defined steps and, similarly, the migration of DENDRITIC CELLS, are also controlled by various CYTOKINES (Tab. 7).

Among these, the protein family of CHEMOKINES plays a pivotal role [45, 46] (Tab. 8). CHEMOKINES are small proteins, mostly with molecular masses between 8 and 10 kDa, with four characteristic cysteine residues forming intracellular bonds. More

than 40 CHEMOKINES have been cloned in human beings. According to their structure, which is also represented at the genomic level by gene clusters, four groups can be distinguished. CXC CHEMOKINES, now termed CXCL 1–15 (L stands for LIGAND, in which the cysteines are separated by an arbitrary amino acid, X), are predominantly chemotactic for NEUTROPHILS and some – interestingly forming a small separate sub-cluster – for T LYMPHOCYTE subsets. CC-CHEMOKINES, CCL1–27, mostly attract MONOCYTES/MACROPHAGES (again with a sub-cluster for LYMPHOCYTES) and XC CHEMOKINES, XCL1 and 2 (or CL1 and 2), attract LYMPHOCYTES. One member of a CX3C family also exists. The CHEMOKINE families bind with overlapping patterns to selective RECEPTOR families of which five are known for CXC ligands, CXCR1–5, ten for CC ligands, CCR 1–10, and one XCR1 or CX3R1, which all belong to the family of G protein-coupled RECEPTORS. CHEMOKINES have fundamental roles in development, homeostasis and function of the IMMUNE SYSTEM. Within the IMMUNE SYSTEM (which is the scope of this chapter) they can be divided into two categories: homeostatic and inflammatory. The homeostatic CHEMOKINES are constitutively expressed regulating the structural organization and cellular composition of peripheral LYMPHOID ORGANS, such as lymph nodes. They also govern the recirculation of LYMPHOCYTES. The inflammatory CHEMOKINES are strongly up-regulated by pro-inflammatory stimuli, predominantly in cells of the IMMUNE SYSTEM (MACROPHAGES, T LYMPHOCYTES), but also in fibroblasts, and participate in the development of inflammatory and immune reactions. This function is supported by the fact that these CHEMOKINES, in addition to their chemotactic properties, are also potent activators of their TARGET cells.

Pharmacological implications

Their strong pro-inflammatory properties make CHEMOKINES candidate drugs. Thus, quite a number of CHEMOKINES have shown antitumor effects in various tumor models [47]. Their participation in inflamma-

TABLE 7. PARTICIPATION OF CIRCULATING LEUKOCYTES IN LOCAL INFLAMMATION

Reaction step	Cytokines involved	Main target cells
Chemotaxis	IL-8 (CXCL8)	Neutrophils
	Eotaxin (CCL11)	Eosinophils
	MCP-1 (CCL2) RANTES (CCL5)	Monocytes
	Lymphotactin (CL1)	Lymphocytes
Emigration from the blood	IFN-γ IL-1 TNF	Endothelial cells: Expression of cell adhesion molecules such as ELAM-1, ICAM-1,VCAM-1
Cell activation	IFN-γ IL-1 TNF	Monocytes/macrophages,
	CSFs IL-8 (CXCL8)	Granulocytes
Expansion of cells	CSFs	Myeloid precursor cells

CL,CCL,CXCL, chemokines; CSF, colony stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor

Table 8. Selected chemokines[1]

		(synonyms)
CXC chemokines	CXCL1	(Gro-α)
	CXCL4	(PF4)
	CXCL8	(IL-8)
	CXCL10	(IP-10)
	CXCL12	(SDF-1α/β)
XC chemokines	XCL1 (CL1)	(Lymphotactin)
CX3C chemokines	CX3CL1	(Fractalkine)
C-C chemokines	CCL2	(MCP-1)
	CCL3	(MIP-1α)
	CCL4	(MIP-1β)
	CCL5	(RANTES)
	CCL11	(Eotaxin)

[1] nomenclature: C, cysteine residues separated by none, one (X), two (X2) or three (X3) arbitrary amino acids; L, ligand (of chemokine receptors)

tory diseases, on the other hand, makes them targets for specific interventions [48,49]. Encouraging results have been achieved in several experimental setups of INFLAMMATION. Progress is hampered, however, by the great redundancy of the CHEMOKINE/CHEMOKINE RECEPTOR families.

The chemokine RECEPTORS, CCR5 and to a lesser extent CXCR4, have raised great interest as CO-RECEPTORS (in addition to CD4) for the entry of HUMAN IMMUNODEFICIENCY VIRUS (HIV) into MACROPHAGES and T LYMPHOCYTES. Recently, the low molecular weight CCR5 CO-RECEPTOR blocker, Maraviroc, was approved for the treatment of HIV infections and AIDS [27] (see chapter C7).

Inhibition of cytokines

Pathophysiology

Because of their multiple functions in the inflammatory process, CYTOKINES offer useful targets for therapeutic intervention [50–55]. While nearly each feasible intervention is going to be evaluated in clinical trials, a number of innovative drugs have been approved within the last few years for indications such as RHEUMATOID ARTHRITIS and inflammatory bowel diseases, or the prevention of transplant rejection. Suppression of immune and inflammatory reactions can be achieved by inhibition of CYTOKINES in several ways [51]: (1) the inhibition of CYTOKINE synthesis, (2) the decrease of CYTOKINES in free active form, (3) the blocking of the interaction with their RECEPTOR, or (4) the inhibition of CYTOKINE-dependent signaling (Fig. 6).

Pharmacological implications

For each mechanism, at least one clinically relevant example exists (Tab. 9). By far the most predominant TARGET cell for immunosuppressants is the T HELPER LYMPHOCYTE.

Cytostatic drugs such as AZATHIOPRINE decrease the number of (T) LYMPHOCYTES and thereby also of CYTOKINE-producing cells. MONOCLONAL ANTIBODIES directed against T cell EPITOPES in part also work

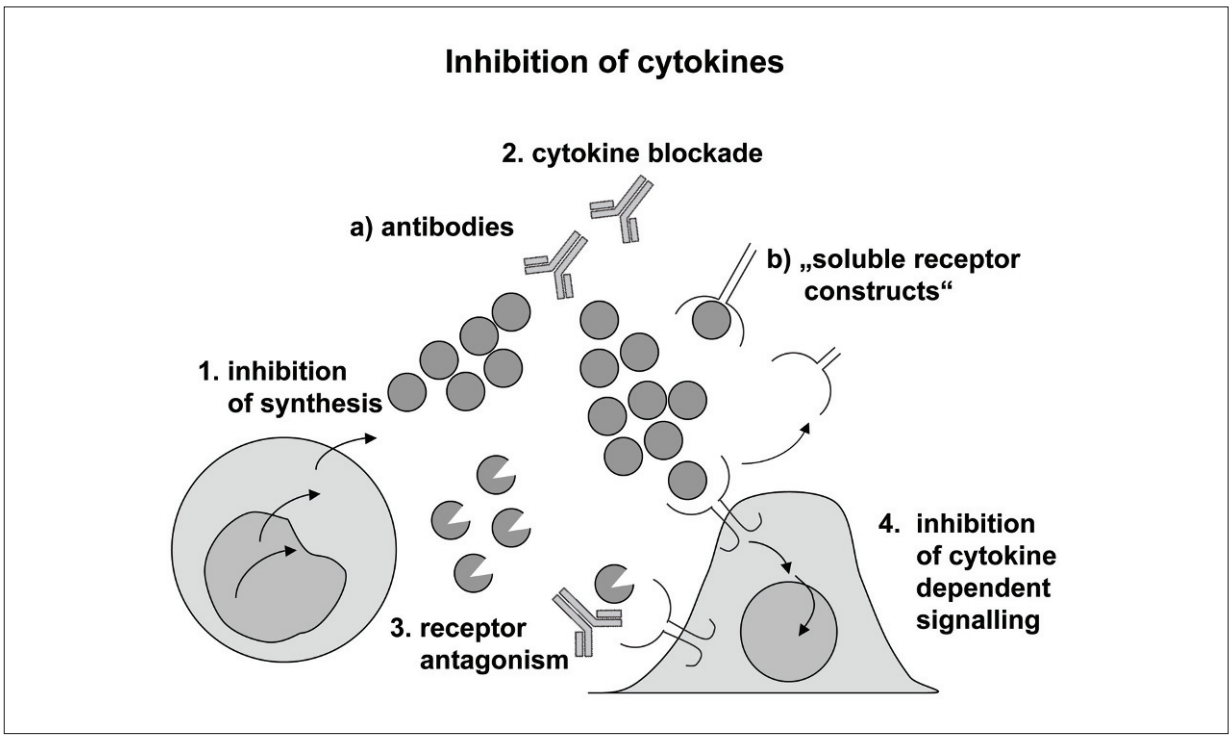

Figure 6. Inhibition of cytokines
●, *cytokine;* ◕, *cytokine antagonist ;* ⅄, *functionally active cytokine receptors;* ⅄, *extracellular domains of ("soluble") receptors*

by this mechanism; antibodies against CD3 or the IL-2-receptor mainly prevent activation or proliferation, respectively. They are effective in preventing transplant rejection and also in the treatment of autoimmune diseases (an example being lupus erythematosus). Without being cytotoxic, by interfering with T cell receptor signaling, modern immunosuppressants, including ciclosporin or tacrolimus, very selectively block the synthesis of T lymphocyte cytokines, predominantly of their growth factor IL-2 (see chapter C12). In a similar way, glucocorticoids (e.g., prednisone) are immunosuppressive by interfering with the gene expression of this cytokine and others synthesized by T lymphocytes [56].

Glucocorticoids also represent the most efficient anti-inflammatory drugs available (see chapter C14). Although they affect multiple pro-inflammatory mechanisms, their efficacy largely relies on the capacity to block gene expression of most pro-inflammatory cytokines, including IL-1 to IL-8, TNF or IFN-γ. A new group of experimental drugs, termed cytokine-suppressive anti-inflammatory drugs (CSAID), has been found, which exhibits a striking selectivity for inhibiting the synthesis of IL-1 or TNF by interfering with critical signal transduction steps. None of these has so far been approved for clinical use. Th2 cytokines such as IL-4 or IL-10 can decrease the synthesis of pro-inflammatory cytokines by down-regulating the activation of their producer cells, such as macrophages (see Fig. 4). Both – predominantly IL-10 – proved to be effective in clinical studies for the treatment of rheumatoid arthritis and other inflammatory diseases [57].

Table 9. Cytokine inhibitors

Mode of action	Example drugs
1. Inhibition of synthesis	
Reduction of the number of cytokine producing cells	
cytostatic immunosuppressants	Azathioprine, leflunomide
monoclonal antibodies to cells	Muromonab CD3
Regulation of cell activity	
regulatory cytokines	(interleukin-4,-10)*
calcineurin inhibitors	Ciclosporin, tacrolimus
Regulation of cytokine gene expression	
glucocorticoids	Prednisone
2. Decrease in concentration of active (free) form	
Monoclonal antibodies against cytokines	
anti-TNF antibodies	Infliximab, adalimumab
anti IL-1 antibodies	Canakinumab
anti IL-12/IL-23 antibodies	Ustekinumab
Soluble cytokine receptors	
soluble TNF receptor constructs	Etanercept
soluble IL-1 receptor constructs	Rilonacept
3. Receptor blockade	
Monoclonal antibodies against cytokine receptors	
antibodies against the IL-2 receptor	Basiliximab
antibodies against the IL-6 receptor	Tocilizumab
Cytokine antagonist	Anakinra
4. Inhibition of cytokine-dependent signaling	Sirolimus
Protein kinase inhibitors	

** Not yet approved, in clinical studies*

Antibodies can block the action of secreted cytokines. A chimeric (mouse/human) antibody directed to TNF-α (infliximab) represented the first example of a specific cytokine-blocking antibody, proving efficacious in rheumatoid arthritis or inflammatory bowel disease [52,53]. infliximab may result – at least in a subgroup of patients – in long-lasting remission and even in reversal of destruction, apparent by X-ray measurements (see chapter C15). infliximab has been followed up to now by three further antibodies, including completely human antibodies (generated by phage display technology) with similar properties (adalimumab, golimumab) and a construct of the Fab' fragment of a humanized antibody coupled to polyethylene glycol (certolizumab-pegol). The major side effect of the therapy with anti-TNF antibodies is an increased risk of infections (as might be expected!), including recurrence of tuberculosis and occasionally septic shock [53]. Very recently, a human monoclonal antibody against IL-1 (canakinumab) has been approved for the treatment of cryopyrin-associated periodic syndromes (CAPS). CAPS are very rare diseases resulting from a spontaneous release of IL-1β due to a gain of function mutation of cryopyrin (NALP3), which is part of an inflammasome processing the inactive precursor molecule to its

released active form, IL-1β (see chapter A7). Several other anti-CYTOKINE ANTIBODIES, including ANTIBODIES against IL-1, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18 or IL-23 are being evaluated in clinical trials for the treatment of several autoimmune or chronic inflammatory diseases [54].

In addition to ANTIBODIES, blocking of free active CYTOKINES can also be achieved in a quite different way. Illustrating the principle of self-limitation, the extracellular domains of many CYTOKINE RECEPTORS are released during immune or inflammatory reactions. As these "soluble RECEPTORS" contain the full CYTOKINE binding site, they bind the free CYTOKINE and thereby dampen its biological effect. A soluble TNF-RECEPTOR construct (ETANERCEPT) – in which "soluble" TNF RECEPTORS were fused to the constant parts of human IgG1 to increase AFFINITY and half-life *in vivo* – has been approved for RHEUMATOID ARTHRITIS and inflammatory bowel disease with similar effectiveness and side effects as INFLIXIMAB. A similar construct of the extracellular portions of the IL-1 RECEPTOR and its accessory protein (rilonacept) was approved for the treatment of CAPS (see above). As a unique example, a naturally occurring antagonist of IL-1, IL-1ra, has been cloned and approved (ANAKINRA) for RHEUMATOID ARTHRITIS. Its predominant side effect is also the increased risk of infections (see chapter C15).

RECEPTORS can be blocked by specific ANTIBODIES. BASILIXIMAB is directed against the IL-2 RECEPTOR and effective as an immunosuppressant in transplanted organ rejection episodes (see chapter C12). Table 10 summarizes indications for inhibitors of CYTOKINES. Preventing the binding of IL-6, a HUMANIZED monoclonal ANTIBODY against the IL-6-RECEPTOR (tocilizumab) was recently approved for the treatment of RHEUMATOID ARTHRITIS.

In the last decade the signal transduction pathways of many cytokine RECEPTORS have been investigated extensively. A core finding was that several phosphorylation cascades exist in which key protein kinases offer targets for pharmacological interventions. Although quite a number of selective protein kinase inhibitors have been found, only very few of them so far have been approved for the prevention of transplant rejection such as sirolimus or everolimus (see chapter C12). Both drugs, by blocking mammalian TARGET of rapamycin (mTOR, a synonym for sirolimus), prevent IL-2-driven proliferation of activated T LYMPHOCYTES. None so far has been approved for the treatment of autoimmune or inflammatory diseases. Some are presently being tested in advanced clinical studies, including inhibitors of the p38 kinase or the MAP-kinases 1 and 2 [58, 59]. Table 10 summarizes the possible indications for cytokine-related anti-inflammatory or immunosuppressive drugs.

Summary and outlook

It was not long after their discovery and subsequent molecular characterization that CYTOKINES were tested for their therapeutic potential. This was only made possible by gene technology, which allowed sufficient amounts to be produced in good quality. Some of them – INTERFERONS and the COLONY-STIMULATING FACTORS – subsequently became established as drugs with great medical and even economic importance. Not all high-flying hopes, however, have been fulfilled, especially with regard to the treatment of malignant tumors. Thus, after a period of set-backs, new strategies have begun to evolve, which allow high local concentrations to be selectively generated, the most sophisticated approach involving the use of genetically altered cells.

On the other hand, CYTOKINES are now known to be crucial participants in the pathogenesis of many diseases. The realization that long-known and valuable drugs, such as the glucocorticosteroids, act predominantly by suppressing the synthesis of certain CYTOKINES, has prompted a search for mechanisms by which the synthesis or function of individual CYTOKINES can be blocked more selectively. Even though CYTOKINES or their inhibitors have developed into indispensable drugs in important indications, it is certain that this is only the beginning. This assumption is based on the growing evidence that these molecules contribute to many more diseases than those anticipated originally; important examples are atherosclerosis, congestive heart failure or neurodegenerative diseases.

Table 10. Potential indications for inhibitors of cytokines

Target cytokine	Indication
IL-1[1]	Autoimmune and chronic inflammatory diseases
IL-2	Organ transplantation, autoimmune diseases
IL-4, IL-13	Allergic (type 1) diseases, especially allergic asthma
IL-5	Allergic asthma (?)
IL-6	Chronic inflammatory diseases, hyperimmunoglobulinemias
IL-8 (and other chemokines)	Chronic inflammatory diseases, autoimmune diseases
IL-12, IL-18	Chronic inflammatory diseases
IL-23	Chronic inflammatory diseases
TNF-α, β	Septic shock, chronic inflammatory and autoimmune diseases
IFN-γ	Autoimmune diseases

IFN, interferon; IL, interleukin; TNF, tumor necrosis factor;
[1] cytokines in bold font represent those for which inhibitors have been approved

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Recommended website (Free online information)

Horst Iblgaufts COPE: Cytokines and cells online pathfinder encyclopedia. *www.copewithcytokines.de* (accessed February 2010)

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Innate immunity – Phagocytes, natural killer cells and the complement system

Timo K. van den Berg, Hergen Spits and Diana Wouters

Introduction

In the second half of the 19th century, Eli Metchnikoff discovered that bacteria can be ingested (phagocytosed) by LEUKOCYTES present in the blood of a variety of different animals. At about the same time, Paul Ehrlich found that certain agents dissolved in blood had bactericidal potential. The scientific discussion on the importance of cellular *versus* humoral factors in our defense against bacteria that followed came to an end when it was recognized that both components enforce each other's effect within the context of the IMMUNE SYSTEM. In 1908, both scientists shared the Nobel Prize for Physiology and Medicine. Further investigations on the nature and the working mechanism of the cells and the proteins that constitute our immunological defense system showed that each of these components is made up of several different constituents. In its turn, this led to the insight that a functional distinction exists between the adaptive and the innate branch of the IMMUNE SYSTEM.

While an INNATE IMMUNE SYSTEM of some form at least appears to be present in all multicellular organisms, the adaptive IMMUNE SYSTEM is present only in jawed vertebrates and therefore seems to be a more recent adaptation that emerged some 500 million years ago. As indicated previously (chapters A2 and 3), the adaptive branch is executed by LYMPHOCYTES, i.e., white blood cells capable of generating ANTIBODIES or performing CYTOTOXIC T cell responses, against foreign structures (ANTIGENS) to the body, and killing virus-infected cells. LYMPHOCYTES are able to differentiate between ANTIGENS that belong to the body and those that are foreign. Importantly, LYMPHOCYTES can display immunological memory: once they have encountered foreign material, they will recognize and respond faster and more efficiently upon subsequent encounters. The INNATE IMMUNE SYSTEM, which does not form immunological memory, is largely responsible for the actual elimination of the source of infection, which may either be viral, bacterial, fungal or parasitic in nature. The major components of the INNATE IMMUNE SYSTEM include PHAGOCYTES, NATURAL KILLER CELLS and the COMPLEMENT SYSTEM. PHAGOCYTES are white blood cells capable of uptake (PHAGOCYTOSIS) and intracellular killing of microbes, in particular after binding of ANTIBODIES and complement proteins to the surface of the microbes (Fig. 1). NATURAL killer (NK) cells are lymphocyte-like cells with CYTOTOXIC potential against certain virus-infected and tumor cells. The COMPLEMENT SYSTEM consists of a series of proteolytic enzymes capable of lysing micro-organisms or marking them for elimination by PHAGOCYTES, often in a fashion that can be further enhanced by specific ANTIBODIES. The activities of these innate systems have to be tightly regulated, because in principle they can not only be harmful to the pathogen, but also to the host itself. This chapter will give a short description of each of these innate systems, their clinical relevance and the potential for therapy in case of failure.

Phagocytes

Many cell types are capable – to some extent – of internalizing and killing micro-organisms, which may sometimes help to eliminate infection. However, MACROPHAGES and GRANULOCYTES are the actual 'professional' PHAGOCYTES, because these cells are equipped with a motile apparatus for actively moving to sites of infection (except for organ-localized MACROPHAGES, the so-called histiocytes), with surface RECEPTORS to bind micro-organisms, granules filled

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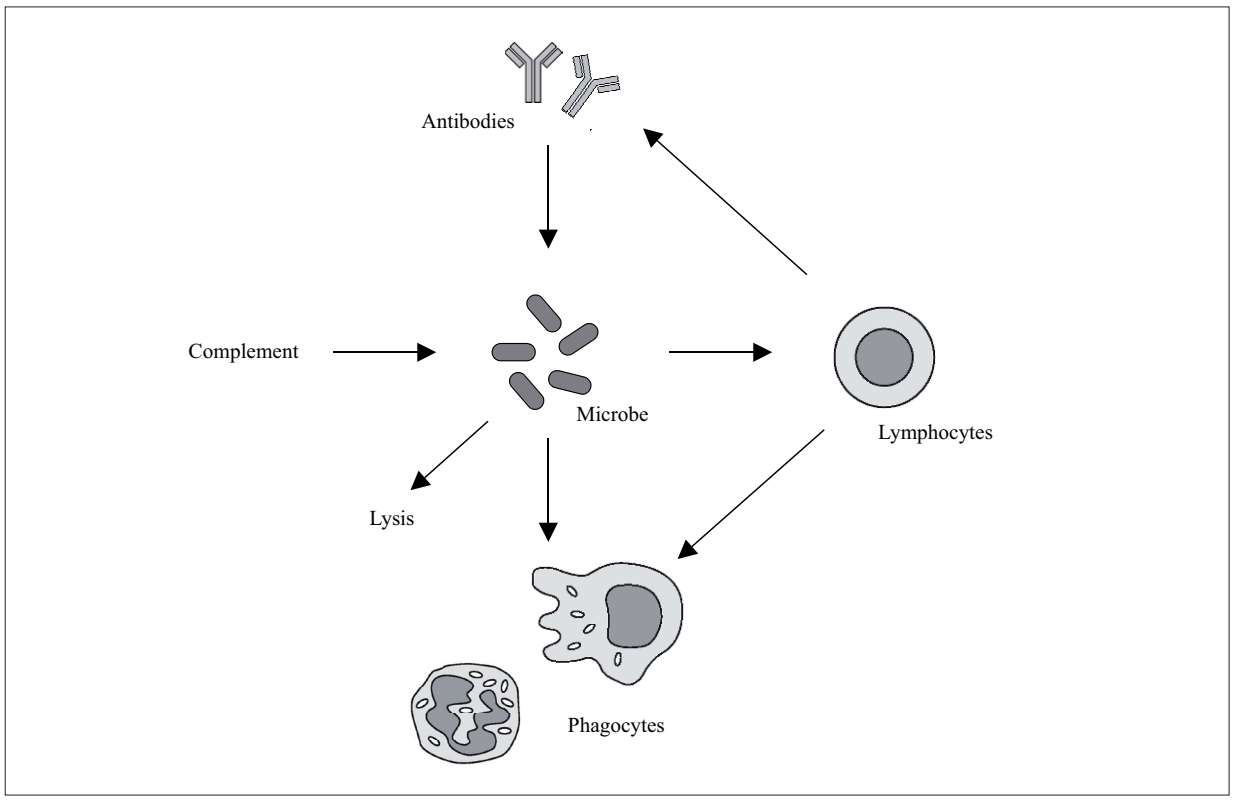

FIGURE 1. COOPERATIVE ACTION OF INNATE AND ADAPTIVE IMMUNE SYSTEMS IN HOST DEFENSE
Microbes can bind antibodies and complement fragments. This may result in direct complement-mediated lysis, phagocytosis by (recruited) macrophages and granulocytes, and/or antigen presentation by dendritic cells to trigger adaptive immune responses that results in the generation of (more) antibodies that may further help to eliminate the pathogen.

with CYTOTOXIC proteins, and with an enzyme that can generate toxic oxygen radicals. MACROPHAGES and GRANULOCYTES are formed in the BONE MARROW from pluripotent hematopoietic precursor cells that differentiate along the myeloid lineage under the influence of growth and differentiation hormones (see chapter A1).

MACROPHAGE progenitors are released into the blood as immature MONOCYTES. These may subsequently traffic to the various tissues and organs where they further differentiate into MACROPHAGES. MACROPHAGES constitute a very HETEROGENEOUS population of cells and this heterogeneity reflects their functional versatility. MACROPHAGES are not only important for host defense, but also play a role in normal tissue development and homeostasis, e.g., by clearing (apoptotic) dead cells.

GRANULOCYTES take about 14 days to develop, and are released into the blood as mature cells. Most GRANULOCYTES become so-called 'neutrophilic' GRANULOCYTES, which exhibit a high anti-microbial potential. Other granulocyte types are 'eosinophilic' GRANULOCYTES, involved in anti-parasite defense, and 'basophilic' GRANULOCYTES, which lack the ability to phagocytose but can release HISTAMINE that plays a role in inflammatory reactions. In the tissues, MACROPHAGES have an estimated life span in the order of weeks-to-months, depending on the subpopulation.

Instead, neutrophilic granulocytes survive only 1–2 days after release from the bone marrow (4–6 days under inflammatory conditions). Thus, neutrophilic granulocytes (neutrophils) need to be formed in much larger numbers than macrophages for efficient surveillance against micro-organisms. Indeed, in healthy adults about 10^{11} neutrophils are newly generated from the bone marrow each day, and this can increase a further tenfold during infection. Macrophages are formed at not more than 10^9 per day. Phagocytes end their life either through necrosis as a result of phagocytosis and the release of toxic mediators, which becomes apparent as pus formation, or through apoptosis (programmed cell death) and subsequent removal by macrophages.

The critical importance of phagocytes for host defense against micro-organisms is illustrated by the recurrent, life-threatening infections that occur in patients with a genetic or acquired shortage or deficiency of these cells. Patients with a shortage of neutrophils, e.g., in a condition known as neutropenia, may be treated with relevant growth factors, such as granulocyte colony-stimulating factor (G-CSF), to compensate for their apparent lack. Complete cure may be achieved by bone marrow transplantation.

Phagocyte mobility

Neutrophils and monocytes have the ability to move actively to the site of an infection. This is caused by the release in these areas of so-called chemotactic substances, small molecules of bacterial or host origin that diffuse into the surroundings and can bind to specific receptors on the phagocytes. Phagocytes are able to 'sense' concentration gradients of these chemotactic agents and to move towards the source of these agents at the site of infection. This process is called chemotaxis. However, phagocytes in the blood must first pass the blood vessel wall before moving into the tissues [1]. This multistep process of extravasation is initiated by reversible interaction of L-selectin, an adhesion molecule on the surface of leukocytes that interacts with carbohydrate structures on endothelial blood vessel cells, and by similar interaction between E-selectin on the endothelial cells with carbohydrate structures on leukocytes. Under normal conditions, this rolling of phagocytes over the endothelium leads to stable adhesion and spreading of the phagocytes on the vessel wall, a process in which integrins play a decisive role (Fig. 2). Finally, diapedesis (transendothelial migration) and movement of the phagocytes into the tissues takes place. In infected or inflamed areas, these processes are strongly increased by the formation of complement fragment C5a, by formylated bacterial peptides, by leukotriene LTB4 and by other chemotaxins. These agents, which are detected by distinct G protein-coupled seven-transmembrane (7TM)-spanning receptor family members, trigger an increase in expression of adhesion proteins on the surface of the phagocytes, such as integrins. Moreover, the integrins are also "activated" by means of a change in their configuration, which causes a higher affinity for their endothelial counter-structures and supports their recruitment to inflammatory sites. In addition, microbial components, like bacterial endotoxin, induce local macrophages to produce interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). These cytokines, as well as endotoxin itself, activate the local endothelial cells to up-regulate the expression of intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular adhesion molecule-1 (VCAM-1), which strongly enhances phagocyte adhesion. Endothelial cells also produce platelet-activating factor (PAF) and IL-8 under these conditions, which remain bound to the endothelial cells and stimulate phagocyte recruitment.

In the process of diapedesis, the endothelial cells that form the blood vessel wall participate actively. Binding of leukocytes to ICAM-1 or VCAM-1 on the endothelial cells induces signaling in the endothelial cells that leads to relaxation of endothelial cell-cell junctions, especially by disrupting the intercellular interactions mediated by, for example, cadherins. This enables the leukocytes to squeeze themselves between two adjacent endothelial cells. The migratory process itself is governed by interactions between the leukocytes and the endothelial cells, mediated for instance by PECAM-1 (CD31), in the intercellular cleft between two endothelial cells (Fig. 2).

Changes in the composition of the extracellular matrix, induced by transforming growth factor-β (TGF-β), and generation by macrophages, endothelial cells

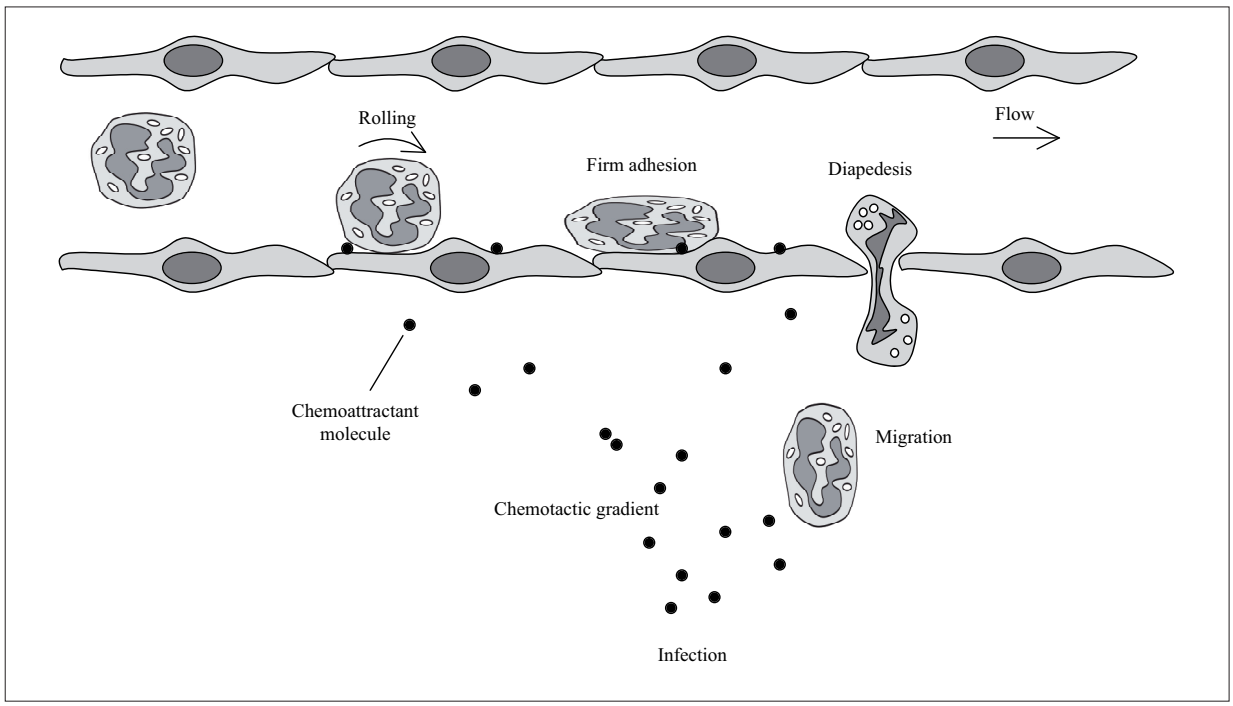

Figure 2. Phagocyte recruitment during inflammation

The process of phagocyte extravasation involves a series of events. The initial interaction with the vessel wall is termed 'rolling' and this is mediated by interactions between lectins and their carbohydrate ligands. In particular, (i) L-selectin is expressed on the phagocyte, and sialyl Lewis-X carbohydrates are expressed on a variety of surface protein carrier molecules on endothelial cells, and (ii) E-selectin is expressed on endothelial cells and sialyl Lewis-X on phagocytes. Chemoattractant molecules (black dots) produced by the inflamed tissue and immobilized by the endothelium then induce activation of integrins on the phagocytes. This results in firm adhesion of these molecules to ICAM-1, ICAM-2, VCAM-1, that are in turn up-regulated on the endothelium by inflammatory cytokines. This is followed by the diapedesis of the phagocytes through the endothelial layer and their migration to the source of inflammation.

and fibroblasts of additional chemotactic CYTOKINES, termed CHEMOKINES, add to the influx of PHAGOCYTES into inflamed tissues. This influx is phagocyte specific, because the CHEMOKINES of the C-C chemokine family, of which monocyte chemotactic protein-1 (MCP-1, CCL2) is the prototype, primarily attract MONOCYTES and MACROPHAGES, whereas CHEMOKINES of the C-X-C family, such as IL-8 (CXCL8), mediate the recruitment of NEUTROPHILS and EOSINOPHILS. In tissues, PHAGOCYTES migrate by a co-ordinated and dynamic process of local attachment to extracellular matrix proteins, propagation of the cell over the fixed area, followed by dissociation of the rear end of the cell.

The biological significance of ADHERENCE and migration is clearly demonstrated by the clinical symptoms of patients with leukocyte adhesion deficiency type 1 (LAD-1). This condition is caused by a genetic defect in β2-INTEGRINS, and is associated with serious recurrent bacterial infections, retarded wound healing, persistent leukocytosis, and a strong deficiency in the generation of inflammatory reactions. In view of the high incidence of death in LAD-1 patients, aggressive management of infections is required. The use of prophylactic treatment with trimethoprim-sulfamethoxazole antibiotics appears beneficial, and when a suitable donor is available,

BONE MARROW transplantation may provide a permanent solution.

Recognition of pathogens by phagocytes

PHAGOCYTES are specialized in uptake and intracellular killing of a large variety of bacteria, yeasts, fungi and mycoplasmata. Unlike cells of the adaptive IMMUNE SYSTEM, which recognize ANTIGENS *via* an immense REPERTOIRE of RECEPTORS generated by somatic RECOMBINATION from a set of encoded gene segments, cells of the INNATE IMMUNE SYSTEM depend on the use of products of a limited number of germ-line encoded RECEPTORS that have evolved to recognize relatively conserved microbial components, termed PATHOGEN-ASSOCIATED MOLECULAR PATTERNS (PAMPs). The RECEPTORS used by cells of the INNATE IMMUNE SYSTEM to recognize "non-self" are collectively termed PATTERN RECOGNITION RECEPTORS (PRRs) [2]. Examples of PAMPs are LIPOPOLYSACCHARIDE (LPS), β-glucan and PEPTIDOGLYCAN (PGN). PRRs transmit signals that can lead to generation of INFLAMMATORY CYTOKINES and CHEMOKINES and to activation of microbicidal systems, such as the production of REACTIVE OXYGEN SPECIES (ROS) and the release of ANTIMICROBIAL PEPTIDES. PHAGOCYTES and sometimes also other cells express a number of PRRs (Tab. 1) that can influence their activation status, cytokine secretion and life-span.

Among the best characterized families of PRRs are the members of the TOLL-LIKE RECEPTORS (TLRs). TLRs are transmembrane proteins consisting of an extracellular leucine-rich repeat (LRR) for the binding of PAMPs and a cytoplasmic tail that is responsible for the signal transduction after ligation of these RECEPTORS. TLR are very conserved among animals and the prototypic TLR molecule, Toll, was originally described in the fruit fly *Drosophila*. Nine different TLRs have been identified within the human IMMUNE SYSTEM and various microbial PAMP ligands have been identified for each of these (Tab. 1). TLRs are expressed on cells of the innate and the adaptive IMMUNE SYSTEMS, but also on other cells. TLRs on the antigen-presenting DENDRITIC CELLS (see chapter A4) act as sensors for PRR, in this context also known as "danger" signals, which trigger the expression of COSTIMULATORY MOLECULES. COSTIMULATORY MOLECULES on antigen-presenting cells, in addition to antigenic stimuli in the form of peptide in association with MHC molecules, provide a critical second signal for the activation of T cells. TLRs are expressed not only on the surface of PHAGOCYTES, enabling these cells to respond to PAMPs present in the extracellular milieu, but also on the phagosomal membrane that surrounds ingested microbes (Tab. 1), suggesting that PHAGOCYTES have the ability to "sense" the contents of the PHAGOSOME.

TLRs share part of their signal transduction pathway with the IL-1 RECEPTOR (IL-1R) family. Stimulation of both types of RECEPTOR ultimately leads to activation of the transcription factor NF-κB, but also to activation of c-jun N-terminal kinase (JNK) and p38 MITOGEN-activated protein kinase (MAPK) (Fig. 3). This cascade induces the expression of pro-INFLAMMATORY CYTOKINES and the differentiation of various immune cells into EFFECTOR CELLS. In addition to this general response to ligation of any TLR, responses exclusive to certain types of TLR also exist, including for instance interferon-α/β (IFN-α/β) production after TLR3 and TLR4 ligation. In PHAGOCYTES, this pathway is involved in migration, DEGRANULATION and NADPH OXIDASE activation (see next section of this chapter), three functions that are essential for proper finding and killing of microbes. These partially different effects of TLR signaling enable the IMMUNE SYSTEM to react in a specific way to various pathogens and to orchestrate the host response for efficient elimination of pathogen.

As opposed to the TLRs that are expressed on the plasma membrane, members of the NOD-LIKE RECEPTOR (NLR), such as NLR3, appear to act as intracellular sensors for infection. Together with other cytosolic components, including the protease caspase-1 and the adaptor protein ASC, they form protein complexes known as INFLAMMASOMES, which upon LIGAND recognition by NLRs activate caspase activity and mediate cleavage and secretion of members of the IL-1 cytokine family that are synthesized as inactive precursors [3]. Excessive activation of the NLR3 pathway occurs in gout where uric acid crystals are formed that provide a strong stimulus for INFLAMMASOME activation, for instance, in the joints. In addition, there are a number of rare genetic autoinflammatory disorders characterized by episodes of fever and INFLAMMATION that are caused by activating

Table 1. Pattern recognition receptors on phagocytes and other cells

	Localization	Ligand	Origin of the Ligand
Membrane pattern recognition receptors			
Toll-like receptors (TLR)			
TLR1 (dimer with TLR2)	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, host
TLR3	Endolysosome	dsRNA*	Virus
TLR4 (with MD2)	Plasma membrane	Lipopolysaccharide	Bacteria, viruses, host
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6 (dimer with TLR2)	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Phagosomal membrane	ssRNA	Virus, bacteria, self
TLR9	Phagosomal membrane	CpG-DNA	Virus, bacteria, protozoa, host
TLR10	Phagosomal membrane	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
NOD-like receptors (NLR)			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	Muramyl dipeptide	Bacteria
NLR1-14	Cytoplasm	Unknown	Microbes, host
RIG-like receptors (RLR)			
RIG-I	Cytoplasm	Short dsRNA, 5′ triphosphate dsRNA	RNA viruses, DNA virus RNA viruses (Picornaviridae)
MDA5	Cytoplasm	Long dsRNA	RNA viruses
LGP2	Cytoplasm	Unknown	
C-type lectin receptors (CLR)			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
Mannose receptor	Plasma membrane	Mannose	Fungi, bacteria
DC-SIGN	Plasma membrane	Mannose	Bacteria, virus
Scavenger receptors			
SR-A	Plasma membrane	Polyanions	Bacteria, host
MARCO	Plasma membrane	Polyanions	Bacteria, host
CD163	Plasma membrane	Unknown	Bacteria, host
Secreted PRRs			
C-type lectins			
Mannose-binding lectin		Mannose	Fungi, bacteria
Ficolin			?
Pentraxins			
C-reactive protein		Phosphorylcholine	?
SAP		Phosphorylcholine	
PTX3		?	
LPS-binding protein (LBP)		Lipopolysaccharide	Gram-negative bacteria

*Abbreviations: *ss/dsRNA/DNA, single-strand/double-strand RNA/DNA*

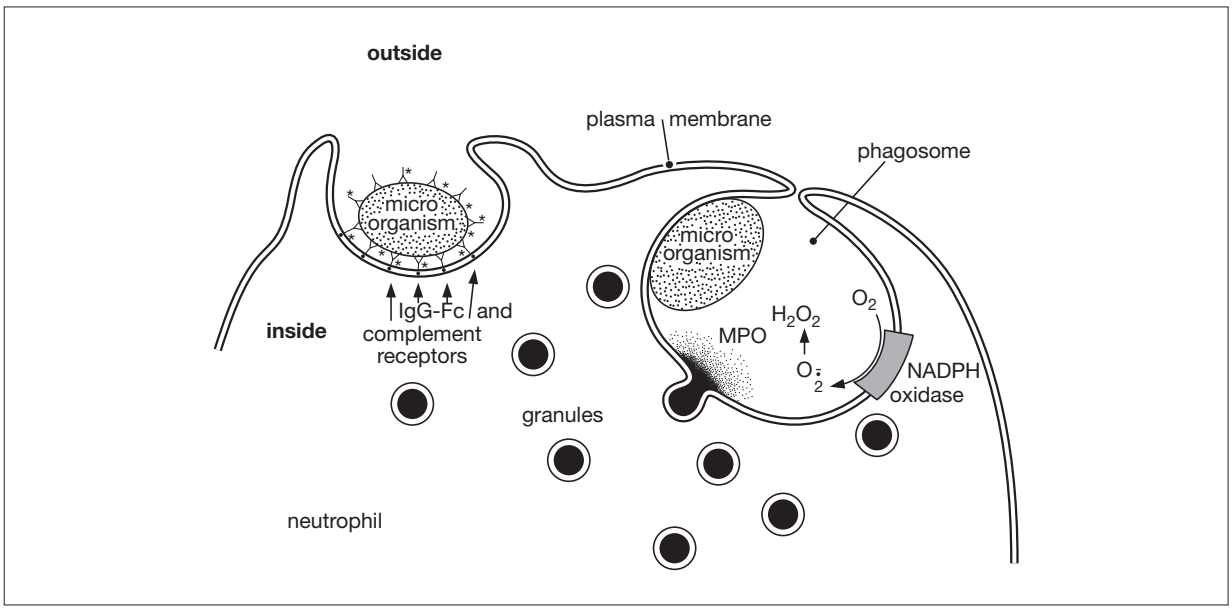

FIGURE 3. SIGNAL TRANSDUCTION PATHWAYS IN TLR SIGNALING

TLR2 forms heterodimers with either TLR1 or TLR6, the other TLRs act as monomers. The cytoplasmic tails of the TLRs contain a TIR domain also present in the adaptor protein MyD88. Upon ligand association with the TLRs, these TIR domains associate, thus coupling MyD88 to the activated TLRs. This leads to recruitment of the IRAK-1 protein kinase via the death domain in both MyD88 and IRAK-1. This recruitment is mediated by the Tollip protein. IRAK-1 is then phosphorylated, detaches from MyD88 and subsequently binds to and activates a protein called TRAF-6. TRAF-6 triggers the activation of MKK6 and TAK1, which in turn activate JNK, p38 MAPK and NF-κB, respectively. This general signal transduction route induces the expression of a set of genes that cause the induction of inflammatory cytokines and the differentiation of various cell types into effector cells. In addition, the MyD88 adapter protein, Mal-TIRAP, is involved in signaling through all TLR except TLR3, 7 and 9. In addition, TRIF, another protein with a TIR domain, is involved in IFN production via TLR3 and TLR4. Figure reproduced with permission from [27]. TIR, Toll/IL-1 receptor; IRAK, IL-1 receptor-associated kinase; Tollip, Toll/IL-1R-interacting protein; TRAF, TNF receptor-associated factor; MKK, MAPK kinase kinase; JNK, c-jun N-terminal kinase; Mal, MyD88-adapter-like; TIRAP, TIR adapter protein; TRIF, TIR domain-containing adapter inducing IFN-β.

mutations in NRL3 [4]. In line with the important role of INFLAMMASOMES and IL-1β production, therapy with ANAKINRA, an IL-1 RECEPTOR antagonist, has shown to be effective in these situations.

Phagocytosis and killing of micro-organisms

Most micro-organisms can only be ingested efficiently after being covered with specific ANTIBODIES and/or complement fragments (a process called OPSONIZATION). ANTIBODIES bind with their variable regions to microbial ANTIGENS and their exposed ANTIBODY FC REGIONS promote activation of the classical complement pathway and the deposition of complement cleavage fragments such as C3b and iC3b onto the microbial surface (see below and Fig. 4). The ANTIBODY FC REGIONS and the complement fragments can then bind to FC RECEPTORS and COMPLEMENT RECEPTORS, respectively, which are expressed on the phagocyte surface. This binding of opsonized micro-organisms to the PHAGOCYTES initiates three reactions in these

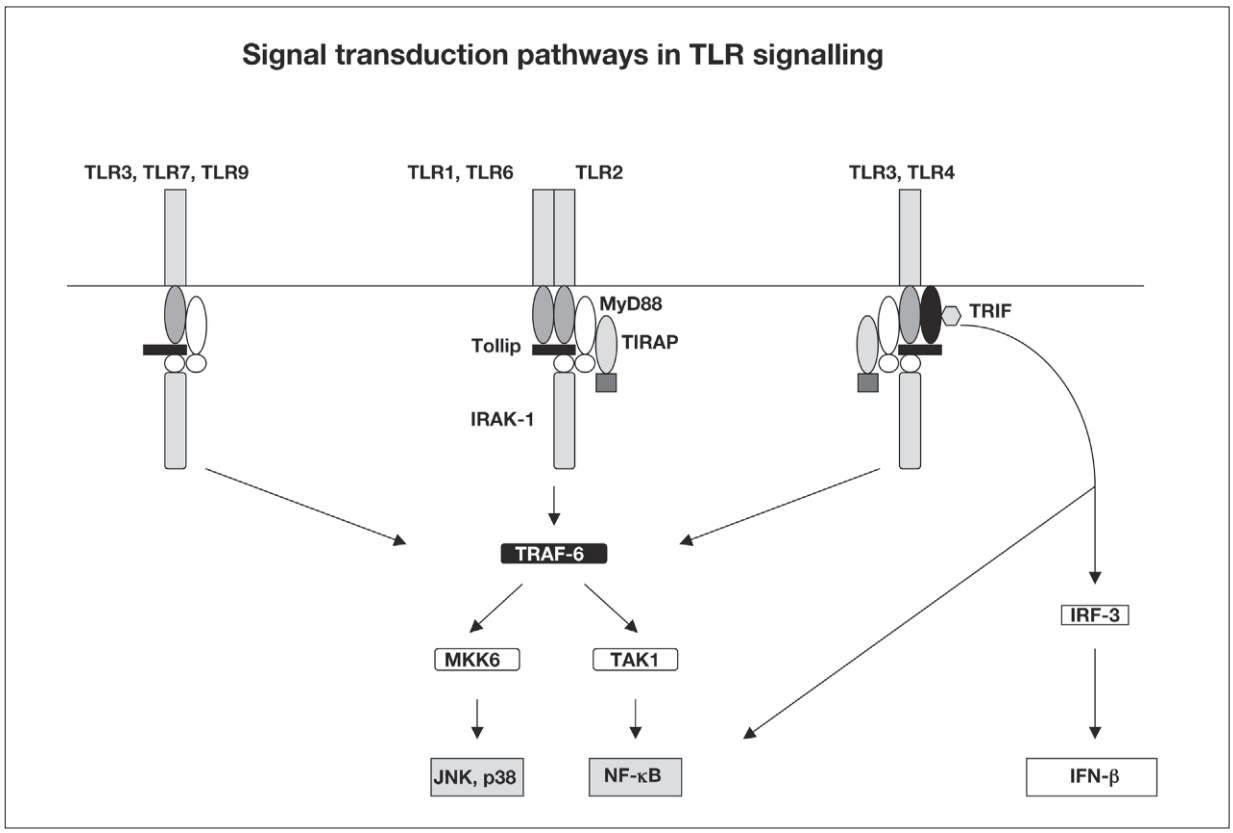

FIGURE 4. SCHEMATIC REPRESENTATION OF PHAGOCYTOSIS, DEGRANULATION AND GENERATION OF REACTIVE OXYGEN PRODUCTS
*MPO, myeloperoxidase; *, complement fragments C3b or iC3b. Figure reproduced with permission from [26].*

cells: (1) rearrangement of cytoskeletal elements that result in folding of the plasma membrane around the microbes (i.e., PHAGOCYTOSIS); (2) fusion of intracellular granules with this PHAGOSOME (i.e., DEGRANULATION) and (3) generation of ROS within the PHAGOSOME (Fig. 4) by the phagocyte. The intracellular granules contain an array of microbicidal proteins, such as serine proteases, acid hydrolases, DEFENSINS, bactericidal permeability-increasing protein (BPI) and myeloperoxidase, as well as a number of microbiostatic proteins, such as metalloproteases, lactoferrin and vitamin B12-binding protein [5]. In NEUTROPHILS, these proteins are divided among several distinct types of vesicles, i.e., azurophilic, specific and secretory granules. Simultaneously with the fusion of the granules with the phagosomal membrane, the NADPH OXIDASE enzyme in this membrane is activated. This enzyme complex, which is assembled from a number of membrane and cytosolic subunits upon appropriate phagocyte stimulation, pumps electrons donated by NADPH in the cytosol into the PHAGOSOME, which combine with molecular oxygen to form superoxide (O_2^-), an anion radical with high reactivity. This sudden increase in oxygen consumption is known as the RESPIRATORY BURST. To compensate for the negative charge delivered to the PHAGOSOME, protons and other cations are also pumped into the PHAGOSOME (Fig. 5). The influx of potassium ions is also instrumental in the release of CYTOTOXIC proteins from the proteoglycan matrix of the azurophil granules. The superoxide product of the NADPH OXIDASE enzyme is spontaneously

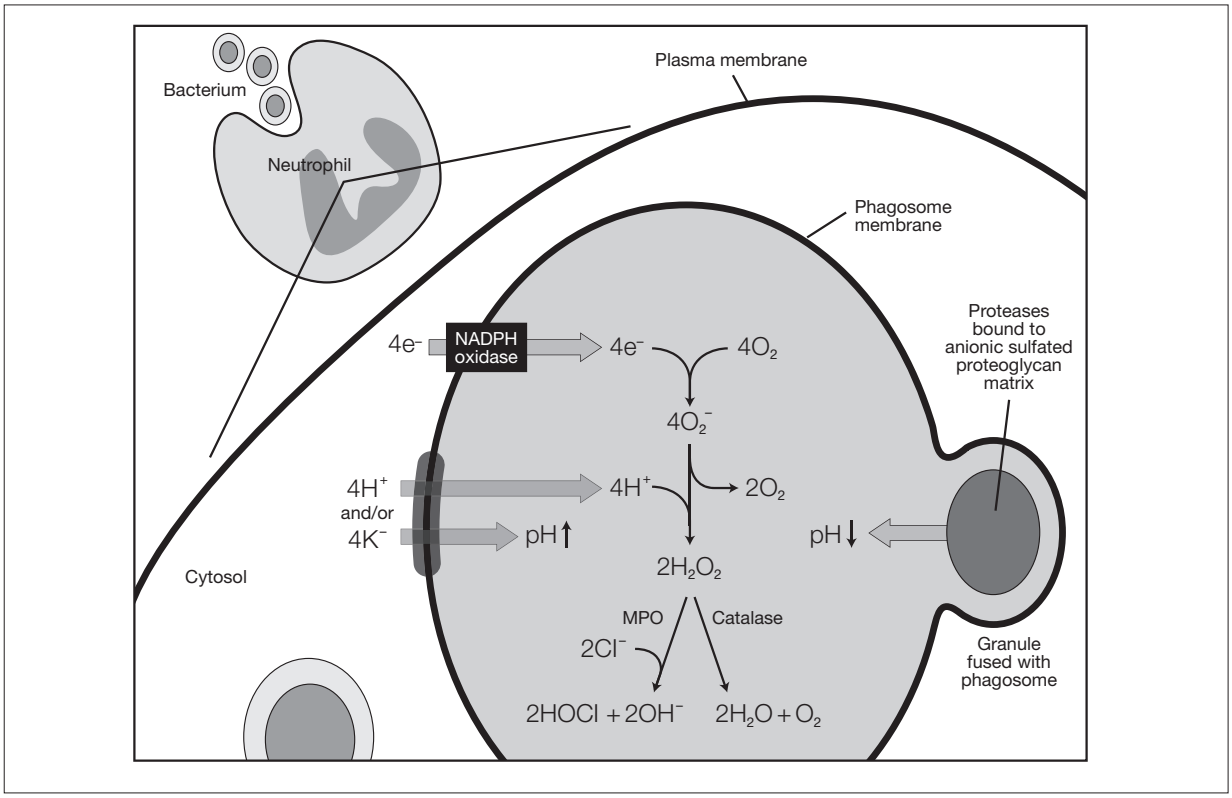

Figure 5. Reactions in the phagosome

The active NADPH oxidase transports electrons across the phagosomal membrane into the phagosome, where the electrons combine with molecular oxygen to generate superoxide (O_2^-). The resulting charge separation is largely compensated for by protons (H^+), which are transported by a voltage-gated channel into the phagosome. However, this charge compensation by protons is not total, because the intra-phagosomal pH rises during the first few minutes after phagosome formation. Potassium ions (K^+) also enter the phagosome, and these ions are instrumental in releasing proteases from their proteoglycan matrix in the azurophil granules that have fused with the phagosome. Superoxide combines with protons to form hydrogen peroxide (H_2O_2), which can combine either with chloride ions (Cl^-) in a myeloperoxidase (MPO)-catalyzed reaction to hypochlorous acid (HOCl), or with another molecule of H_2O_2 in a catalase-mediated reaction to water and molecular oxygen. Figure reproduced with permission from [28].

converted into hydrogen peroxide, which then reacts with chloride anions in a myeloperoxidase-catalyzed reaction to form hypochlorous acid ($HOCl^-$). This last product is a very reactive substance and very toxic for many bacteria and fungi. HOCl can also react with primary and secondary amines to form *N*-chloramines, which are as toxic as HOCl but much more stable. Thus, the NADPH OXIDASE enzyme is essential in the microbicidal action of PHAGOCYTES, both by liberating proteolytic enzymes and by generating reactive oxygen compounds.

The biological significance of the microbicidal apparatus of PHAGOCYTES is again illustrated by the consequences of its failure. Patients with chronic granulomatous disease (CGD), whose PHAGOCYTES lack an active NADPH OXIDASE, suffer generally already at an early age from very serious infections caused by catalase-positive micro-organisms (cat-

alase-negative organisms themselves secrete some hydrogen peroxide, which can be used by CGD PHAGOCYTES to kill these organisms). Patients with a deficiency of specific granules (a very rare disorder) suffer from recurrent infections with various microbes. Patients with the syndrome of Chédiak-Higashi are characterized by NEUTROPENIA and recurrent infections with purulent micro-organisms. The PHAGOCYTES (and many other cell types) of these patients contain aggregated granules, which decrease cell mobility and DEGRANULATION. Infections in CGD and Chédiak-Higashi patients are treated with intravenous antibiotics and surgical drainage or removal of resistant infections. Prophylactic treatment with trimethoprim-sulfamethoxazole is very successful. In addition, prophylaxis with high doses of vitamin C in Chédiak-Higashi patients and with IFN-γ in CGD patients may also be beneficial. BONE MARROW transplantation is at present the only curative therapy.

Inflammatory reactions

PHAGOCYTES also play a critical role in inflammatory responses, e.g., by presenting microbial ANTIGENS to LYMPHOCYTES, by releasing inflammatory mediators (chemotactic peptides, LEUKOTRIENES, CYTOKINES), and by removing damaged host cells. Moreover, NEUTROPHILS in particular can also cause tissue damage; this is usually limited to the infectious period and intended to give the PHAGOCYTES access to the infectious agents. However, in chronically inflamed areas, such as those caused by autoimmune reactions, permanent macrophage activation will occur, and neutrophil influx and activation will continue. This will lead to excessive release of toxic substances (i.e., ROS and proteases) from the NEUTROPHILS. Under normal conditions, these proteases are quickly inactivated by serine protease inhibitors (SERPINS such as α1 antitrypsin) and α2-macroglobulin, which are abundantly present in plasma and tissue fluids. During neutrophil activation, however, ROS and elastase released from these cells will inactivate these protease inhibitors. Moreover, the reactive oxygen compounds will also activate metalloprotease precursors, which will then degrade tissue matrix proteins. Figure 6 provides an overview of these reactions. Clearly, when this process is not self-limiting, irreversible tissue damage may result. In addition, SERPINS involved in regulating the complement, coagulation, fibrinolytic and the contact system cascades may also be inactivated, which will further add to the severity of the clinical symptoms. Well-known clinical conditions in which this may happen are septic SHOCK, gout, RHEUMATOID ARTHRITIS, autoimmune vasculitis, some types of glomerulonephritis, adult respiratory distress syndrome, lung emphysema, acute myocardial infarction, burns, major trauma and pancreatitis.

To limit the extent of these inflammatory reactions, PHAGOCYTES, especially NEUTROPHILS, have a very short survival time, because they are programmed to die within a few days after leaving the BONE MARROW by APOPTOSIS. This form of cell death prevents leakage of toxic compounds from the cells into the surroundings but instead leads to surface expression of molecules that induce binding, uptake and degradation of the cells by MACROPHAGES. Moreover, to prevent excessive phagocyte activation, nature has equipped these cells with a number of inhibitory mechanisms to dampen their activities. These include for instance the inhibitory IMMUNORECEPTOR signal regulatory protein (SIRP)α. SIRPα association with the broadly expressed CD47 surface protein causes phosphorylation of its cytoplasmic IMMUNORECEPTOR TYROSINE-BASED INHIBITION MOTIFS (ITIM) and subsequent recruitment of the protein tyrosine phosphatases SHP-1 and SHP-2, which inhibit activatory signaling pathways dependent on tyrosine phosphorylation. Another example is the CD200R IMMUNORECEPTOR, also primarily expressed on PHAGOCYTES, which, upon binding with its LIGAND, the broadly expressed molecule CD200, provides inhibitory signals that suppress phagocyte function. Certain pathogens, such as pox- or herpes- viruses, encode CD200 homologues that, when expressed on virus-infected cells, act to suppress phagocyte functions *via* CD200 triggering and therefore to evade immunity.

Finally, MONOCYTES and MACROPHAGES also contain an inhibitory Fcγ RECEPTOR, FcγRIIb, which, in contrast to the activating Fcγ RECEPTORS, does not contain or associate with proteins with a cytoplasmic IMMUNORECEPTOR TYROSINE-BASED ACTIVATING MOTIF (ITAM), but instead contains again an ITIM. Probably, this FcγRIIb again serves to control phagocyte reactions.

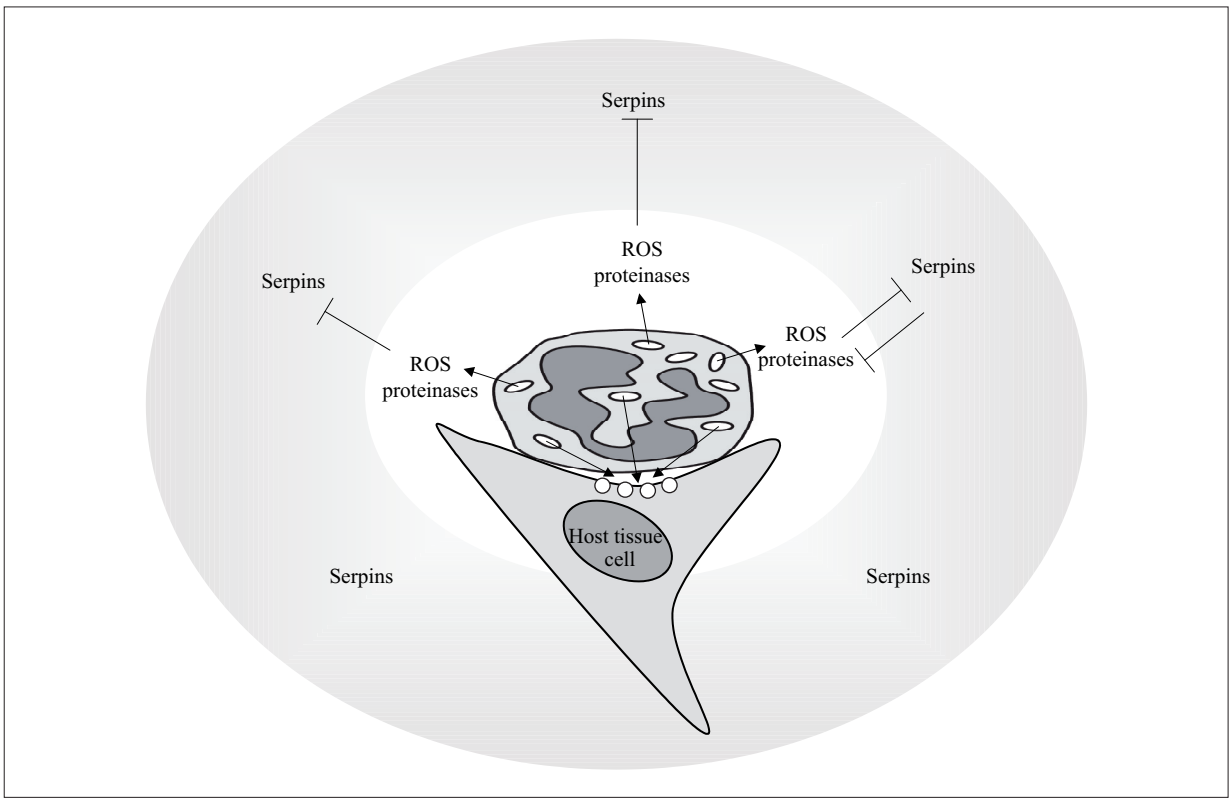

Figure 6. Interplay between oxidative and proteolytic inflammatory reactions

During neutrophil activation, by pathogens for instance, degranulation occurs and proteinases are released. Among these proteinases, serine proteases (elastase, cathepsin G, proteinases 3, urokinase-type plasminogen activator) are released from the azurophilic granules. Normally, these proteinases are inactivated by serine protease inhibitors, collectively termed serpins [α1-proteinase, α2-macroglobulin, α1-chemotrypsin inhibitor and secretory leukoproteinase inhibitor (SLPI)] produced in tissue fluids and plasma. However, elastase and reactive oxygen species (ROS), also produced during neutrophil activation, can inactivate these serpins permitting proteolytic activity towards pathogens, but also against normal tissue. ROS also induce the activation of metalloproteinases, such as collagenase and gelatinase released from specific granules, which in their activated form, can also directly contribute to tissue damage or indirectly via the inactivation of serpins. Thus, ROS and proteases collaborate to inflict tissue destruction during inflammation.

Natural killer cells

NK cells were described for the first time 28 years ago. Operationally, these cells were defined by their ability to kill certain tumor cells *in vitro* without prior contact with these tumor cells. Development of NK cells does not require gene rearrangements as is the case for T lymphocytes, for example, but NK cells are nonetheless developmentally closely related to T lymphocytes (reviewed in [6]). Although clearly derived from hematopoietic stem cells, the anatomical site(s) of differentiation of NK cells is still unknown. cytokines are critical for the development of NK cells both in humans and mice. For instance, NK cells are absent in mice with a deficiency of the gamma chain of the IL-2 receptor and in severe combined immunodeficiency patients who present with

mutations in this gamma chain. The IL-2Rγ chain is shared by RECEPTORS for several CYTOKINES, including IL-15. This latter cytokine appears to be essential for optimal NK cell development [7].

NK cells are implicated in INNATE IMMUNITY against foreign tissue, tumor cells, and microbes such as parasites, intracellular bacteria and viruses [8]. They appear to be important in early phases of the immune response, in which T LYMPHOCYTES are not yet involved. There is convincing evidence that, in man, NK cells are involved in defense against viral infections, in particular against herpes viruses [9]. The mechanisms by which NK cells mediate their effects in infections have not yet been fully elucidated, but it seems likely that CYTOKINES, like IFN-γ, produced by the NK cells are involved. In addition, NK cells can control virus infections by killing virus-infected cells. Excessive activation of NK cells may be deleterious. In animal models for lethal sepsis, it has been shown that elimination of NK cells prevents mortality and improves outcome.

NK cells can mediate ACUTE REJECTION of BONE MARROW grafts [10]. This is not the "raison d'être" of NK cells, of course, but this phenomenon has led to the concept that NK cells recognize cells in which one or more self-MHC class I ANTIGENS is lacking or modified, the "missing self hypothesis", which would explain why normal tissue is protected against NK cell-mediated lytic activity [10].

Cytokine regulation of NK cells and the role of NK cell cytotoxicity in immunity against infections

NK cells are intermingled in an intricate cytokine network; they respond to and produce CYTOKINES that play a role in immunity against infections [7, 9, 11]. NK cells respond to IL-15 produced by MONOCYTES, and to IL-12 produced by infected MONOCYTES and DENDRITIC CELLS. These CYTOKINES induce growth of NK cells, and particularly IL-12 induces NK cells to produce IFN-γ rapidly after infection. IFN-γ not only has anti-viral effects itself, but is also a strong inducer of IL-12 production. Moreover, it has been shown convincingly that IFN-γ-activated MACROPHAGES are instrumental in the immune response against certain micro-organisms such as *Listeria monocytogenes* [12]. Furthermore, IL-12 plays an essential role in induction of Th1 LYMPHOCYTES (producing IFN-γ but not IL-4; see chapter A5). Thus, a complex interplay between DENDRITIC CELLS, MACROPHAGES, NK cells and T LYMPHOCYTES ensures high levels of production of IFN-γ and IL-12, amplified through positive feed-back loops. IL-10, a product of MACROPHAGES, LYMPHOCYTES and other cell types, is a strong negative regulator of IL-12 production by PHAGOCYTES and of IFN-γ production by NK cells. IL-10 may be produced relatively late in an immune response, dampening the strong responses induced by IL-12 and IFN-γ.

Activation of NK cells by viral and microbial infections enhances CYTOTOXIC activity. This is mediated by IFN-α/β, produced by virus- or bacteria-stimulated NATURAL IFN-producing cells (IPC), also called plasmacytoid DENDRITIC CELLS (pDC) [13]. Bacteria may induce IFN-α/β production through unmethylated CpG motifs, which are prevalent in bacterial but not in vertebrate genomic DNA. Oligodeoxynucleotides (ODN) containing unmethylated CpG motifs activate host defense mechanisms, leading to innate and acquired immune responses. The recognition of CpG motifs requires TLR9 that is expressed on IPC/pDC [13]. Some microbial infections, however, activate NK CYTOTOXICITY directly without IFN-α/β induction; this appears to be dependent on IL-12 and IFN-γ.

Recognition by NK cells

There are two mechanisms of cell-mediated cytolysis. One is mediated through perforin, a protein secreted by CYTOTOXIC LYMPHOCYTES that forms pores in the membranes of TARGET cells. TARGET cells can also be killed by an interaction of the Fas molecule on the TARGET cell with its LIGAND on the CYTOTOXIC cell. This interaction activates proteases in the TARGET cell, resulting in APOPTOSIS. Clearly NK cells mediate their CYTOTOXIC effects predominantly by a perforin-dependent mechanism, because little NK activity is present in perforin-deficient mice [14]. The remaining NK activity is probably mediated by Fas/FasL interaction [15].

The mechanism of NK cell recognition and the RECEPTORS involved have been elusive for a long time.

Recent studies, however, have provided some insight into the complex way in which NK cells recognize their TARGET cells. It is likely that NK cells do not have one single NK RECEPTOR that accounts for all biological responses, such as cytokine production and CYTOTOXIC activity. Rather, it appears that NK cells utilize a vast array of RECEPTORS that induce their effector functions, which are often counteracted by inhibitory RECEPTORS specific for self-MHC class I ANTIGENS [8]. The positive and negative signaling pathways used by NK cells share many common features with RECEPTORS expressed by T and B LYMPHOCYTES. Signals are transmitted by small transmembrane adaptor proteins that possess the so-called ITAM in their cytoplasmic domains. One of these adaptors, DAP12, associates with numerous NK cell RECEPTORS, including Ly49, CD94/NKG2C and CD94/NKG2E in mice, and in humans with several activating KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTOR (KIR), with CD94/NKG2C and with NKp44. Other adapters, such as CD3ζ and the FcR common γ-chain, associate with the human NK cell RECEPTORS NKp30, NKp46 and CD16 [8]. The RECEPTORS NKp44 and NKp46 appear to bind the hemagglutinin of influenza virus due to the presence of sialic acid on these RECEPTORS [16]. The NKp44 and NKp46 RECEPTORS also recognize ANTIGENS expressed on tumor cells, but the nature of these ligands is unknown.

Another NK cell RECEPTOR, NKG2D, has spurred much interest recently, since it allows NK cells to recognize virus-infected and transformed cells [17]. NKG2D is associated with the adapter DAP10, expressed as a transmembrane-anchored disulfide homodimer. Ligands of NKG2D include cell surface ANTIGENS that are up-regulated on transformed or virus-infected cells, such as MICA and MICB, two MHC-like stress-dependent cell surface ANTIGENS. MONOCLONAL ANTIBODIES against many adhesion, activation or COSTIMULATORY MOLECULES on NK cells are able to activate these cells *in vitro*. These molecules include CD2, CD27, CD28, CD44, CD69, LFA-1 and DNAM-1 [16, 18]. However, whether NK cells are activated through one or more of these RECEPTORS in the responses against infected cells or in graft rejection *in vivo* remains to be determined.

The strong CYTOTOXIC activities of activated NK cells raise the question as to how normal tissue is protected from attack by these cells. A solution to this conundrum came from studies on the phenomenon of hybrid resistance [10]. It was recognized in 1979 that NK cells mediate hybrid resistance to BONE MARROW or tumor grafts. This is a situation in which BONE MARROW or tumor grafts of parental origin (either A or B) are rejected by AxB F1 hosts (A and B designate the MHC genotype). This resistance cannot be mediated by T LYMPHOCYTES, because these cells are tolerant to the A and B MHC ANTIGENS of the parents. It is now clear that NK cells possess a sophisticated system of inhibitory RECEPTORS that account for their ability to reject BONE MARROW grafts that lack some MHC ANTIGENS present on the NK cells themselves. These inhibiting RECEPTORS interact with MHC ANTIGENS (Fig. 7). This feature of NK cells allows them to efficiently kill MHC class I-negative tumor cells and to remove infected cells with down-regulated self-MHC. What is more important, this provides for a mechanism by which normal tissue is protected against cytolytic activity by autologous NK cells. It is assumed that all NK cells express at least one RECEPTOR for self-MHC class I ANTIGENS.

Two groups of these inhibitory MHC binding RECEPTORS have now been identified (Tab. 2) [8]. One group consists of C-type LECTIN molecules and is exemplified by the Ly49 gene family in the mouse. Ly49A is the best characterized gene and encodes a disulfide-bonded homodimer that binds to H-2D and H-2K molecules. As a consequence, TARGET cells that express these MHC ANTIGENS are not lysed by Ly49A-positive NK cells. The Ly49 family may comprise around ten members with different, though overlapping, MHC class I, H-2K and H-2D, specificities (Tab. 2). A second murine inhibitory RECEPTOR is CD94/NKG2A, which recognizes the MHC class I-like molecule Qa-1b. Human homologues of Ly49 genes have not yet been identified. However, there is a human homologue of CD94/NKG2A that recognizes HLA-E, an antigen with limited POLYMORPHISM.

In humans, a second group of inhibitory RECEPTORS appears to function on NK cells that are designated KIRs). KIRs are involved in the recognition of the various types of HLA-A, HLA-B, HLA-C and HLA-G ANTIGENS (Tab. 2). Unlike the Ly49 RECEPTORS, KIR are type-I glycoproteins. They are related to the Ig supergene family and are probably encoded by a small

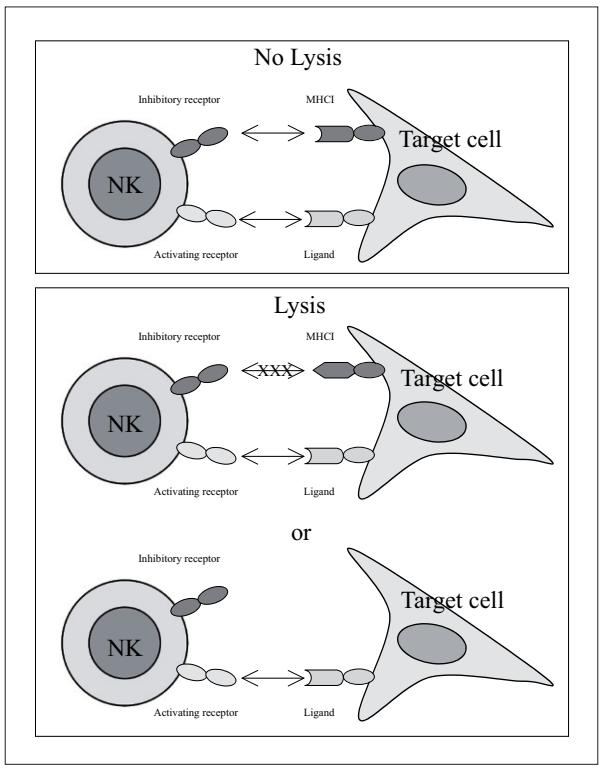

Figure 7. Regulation of NK activity by MHC class I-binding receptors

Lysis by NK cells does not occur when an inhibitory receptor interacts with an MHC class I antigen on the target cell, despite the fact that a cytolysis-activating receptor also interacts with its ligand. NK cells lyse target cells when the interaction between the inhibitory receptor is not triggered, either because the receptor is not specific for the MHC antigen, when this interaction is blocked by antibodies, or when the target cell does not express MHC class I antigens at all.

number of genes. Three different protein isoforms have been described. The KIR recognizing HLA-Bw4 and HLA-A are proteins with three Ig-like domains (KIR3DL), whereas HLA-C-binding KIRs have two Ig-binding domains (KIR2DL). KIR with Ig gene similarity that bind to MHC class I ANTIGENS have not yet been found in mice.

The cytoplasmic domains of all inhibitory NK RECEPTORS possess ITIM. These ITIM are phosphorylated upon engagement of the RECEPTORS and recruit phosphatases to counteract activating signals that induce phospho-kinase activation. The src homology-containing phosphatases SHP-1 and -2 are the predominant tyrosine phosphatases, but some Ly49 RECEPTORS can recruit the SH2 domain-containing inositol phosphatase SHIP.

Table 2. Inhibitory NK cell receptors for MHC molecules

Species	Receptor	Ligand
Mouse	Ly49	H-2K, H-2D
	CD94/NK2A	Qa-1b
Human	KIR2DL2, KIR2DL3	HLA-C1
	KIR2DL1	HLA-C2
	KIR2DL4	HLA-G
	KIR3DL2	HLA-A
	KIR3DL1	HLA-Bw4
	CD94/NKG2A	HLA-E
	CD159a	

The complement system

The COMPLEMENT SYSTEM consists of more than 20 proteins. Most of these are synthesized in the liver and circulate in blood as inactive precursor proteins, also known as COMPLEMENT COMPONENTS. In addition, some complement proteins are expressed as membrane proteins, which serve to dampen undesired activation on cell membranes. During activation, one factor activates the subsequent one by limited proteolysis, and so on [19]. Because this activation process resembles a cascade, the COMPLEMENT SYSTEM is considered as one of the major plasma cascade systems, the others being the coagulation, the fibrinolytic and the contact systems. The physiological role of the COMPLEMENT SYSTEM is to defend the body against invading micro-organisms and to help remove immune complexes and tissue debris.

Hence, deficiencies of complement may predispose to bacterial infections, and immune complex and AUTOIMMUNE DISEASES.

Activation

The COMPLEMENT SYSTEM can be activated by three pathways, which are initiated *via* separate mechanisms and eventually converge in a common TERMINAL PATHWAY. The CLASSICAL PATHWAY consists of the C1 complex (composed of the recognition molecule C1q and the associated proteases C1r and C1s), C4 and C2. The CLASSICAL PATHWAY is activated by binding of C1q to IgG- or IgM-containing immune complexes or other repetitive structures such as the acute-phase protein CRP [20]. The LECTIN PATHWAY essentially uses the same molecules as the CLASSICAL PATHWAY, e.g., C4 and C2, except that its recognition molecules are mannan binding LECTINS (MBL) or ficolins that bind to a wide array of carbohydrate structures on pathogenic surfaces and are associated with the serine proteases MASP-1 and MASP-2. The ALTERNATIVE PATHWAY, consisting of factors B, D and properdin, can be initiated by spontaneous cleavage of complement component C3. AMPLIFICATION of the ALTERNATIVE PATHWAY only takes place on surfaces that are not protected by complement inhibitors. TARGET SPECIFICITY is mainly determined by the carbohydrate environment of bound C3b, which influences the outcome of the competition between factor B and factor H, the major ALTERNATIVE PATHWAY inhibitor. The ALTERNATIVE PATHWAY is not only an activation pathway by itself, but also provides an AMPLIFICATION loop for the other two pathways. C3, which is activated *via* the classical or LECTIN PATHWAY, may initiate the ALTERNATIVE PATHWAY.

The three pathways converge at the level of the third complement component, C3, to proceed as the common TERMINAL PATHWAY, which consists of C5, C6, C7, C8 and C9 (Fig. 8) and forms the MEMBRANE ATTACK COMPLEX. During activation some complement factors, in particular activated C3, covalently bind to the activator, thereby forming ligands for C3 RECEPTORS on phagocytic cells to facilitate PHAGOCYTOSIS. In case activation occurs at a cell membrane, the common pathway proteins (C5b to C9) form macromolecular complexes, consisting of polymerized C9 in addition to C5b, C6, C7 and C8, which insert into the membrane as pores. Insertion of complement pores into cell membranes will allow the exchange of ions and hence induce lysis of the cell. However, under sublytic conditions complement pores do

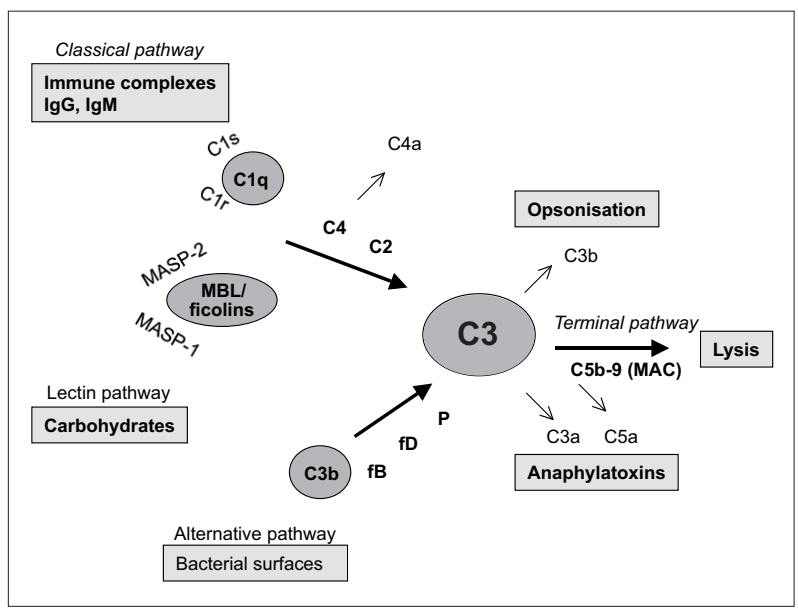

FIGURE 8. COMPLEMENT ACTIVATION PATHWAYS

The complement system can be activated via three pathways; the classical, lectin and alternative pathway that eventually converge into the common terminal pathway.

MBL, mannan binding lectin; MASP, MBL-associated serine protease; fB, factor B; fD, factor D; P, properdin; MAC, membrane attack complex.

not lyse cells, but rather lead to signal transduction and hence to an altered activation state of the cell. In addition, complement pores may induce an exchange between phospholipids of the outer and inner leaflet of the cell membrane (a so-called flip-flop mechanism).

Biological effects

Activation of complement not only fixes some complement proteins onto the activator to promote PHAGOCYTOSIS, it also results in the generation of biologically active soluble peptides and macromolecular complexes [19]. Among these are C5a, C3a and C4a, which are released from C5, C3 and C4, respectively, during activation and – because of their biological effects – are also known as the ANAPHYLATOXINS. For example, C5a, the most potent ANAPHYLATOXIN, is chemotactic for NEUTROPHILS and able to induce aggregation, activation and DEGRANULATION of these cells. In addition, the ANAPHYLATOXINS may enhance vasopermeability, stimulate adhesion of NEUTROPHILS to endothelium, activate platelets and endothelial cells and induce DEGRANULATION of MAST CELLS and the production of the vasoactive eicosanoid THROMBOXANE A2 and the peptidoleukotrienes LTC4, LTD4 and LTE4 by mononuclear cells (see chapter A7). Moreover, they may stimulate or enhance the release of CYTOKINES such as TNF-α and IL-1β and IL-6 from mononuclear cells. The so-called TERMINAL COMPLEMENT COMPLEXES (TCC or C5b–C9), at sublytic concentrations, also induce cells to release mediators, such as CYTOKINES, proteinases and EICOSANOIDS. Finally, complement activation products may induce the expression of tissue factor by cells and thereby initiate and enhance coagulation. Thus, complement activation products have a number of biological effects that may induce and enhance inflammatory reactions (Tab. 3).

TABLE 3. BIOLOGICAL EFFECTS OF COMPLEMENT ACTIVATION PRODUCTS

Complement product	Effect
C5a	Chemotaxis
C5a; C3a	Mast cell degranulation
C5a; C3a	Platelet degranulation
C5a	Phagocyte degranulation
C5a	Stimulation of O_2^- generation by phagocytes
C5a; C5b-9	Enhancement of cytokine release
C5b-9; C5a?	Expression of tissue factor
C5a; C5b-9	Induction of prostaglandin and leukotriene synthesis
C3b; iC3b; C4b	Opsonisation of micro-organisms
C5b-9	Cell lysis
C3b	Enhanced antibody response

Complement regulation

The COMPLEMENT SYSTEM is tightly regulated to prevent excessive activation on a single TARGET, fluid phase activation and activation on host cells. To this end, COMPLEMENT REGULATORY PROTEINS are present in plasma and on host cell membranes. The initiation step of both the classical and the LECTIN PATHWAY is inhibited by the soluble regulator, C1-inhibitor (C1-inh). Other fluid phase complement inhibitors are factor I, factor H and C4bp.

To prevent lysis of innocent bystander cells, host cells are equipped with membrane proteins that inhibit complement activation at various levels. These proteins include the membrane regulatory proteins, decay-accelerating factor (DAF; CD55), membrane cofactor protein (MCP; CD46, not to be confused with the chemokine MCP) and C3b RECEPTOR (CR1), as well as homologous restriction factor (HRF) and membrane inhibitor of reactive lysis (CD59). DAF, MCP and CR1 prevent unwanted activation on membranes by inhibiting the assembly of C3 convertases and accelerating their decay. C3 convertases are macromolecular complexes formed during classical or ALTERNATIVE PATHWAY activation that cleave and activate C3. HRF and CD59 inhibit complement lysis by interfering with the formation of C5b–C9 complexes. Several of these proteins, i.e., DAF, CD59 and

HRF, are anchored to the cell membrane *via* a glycan linkage to phosphatidylinositol. This link is defective in the blood cells of patients suffering from paroxysmal nocturnal hemoglobinuria (PNH). Hence, the red cells of these patients have strongly reduced levels of these complement-inhibiting membrane proteins, and are therefore more susceptible to reactive complement lysis, which largely explains the clinical symptoms of PNH. Furthermore, reduction of membrane regulatory proteins has been found locally in the tissues exposed to complement activation, and this is assumed to contribute to complement-mediated tissue damage in INFLAMMATION. The mechanism of this reduced expression is not clear.

Evaluation of the complement system in patients

The functional state of the COMPLEMENT SYSTEM in patients can be assessed in various ways. The overall activity of the system can be measured by so-called CH_{50} and AP_{50} HEMOLYTIC ASSAYS. In these assays, ANTIBODY-sensitized sheep ERYTHROCYTES (CH_{50}) or non-sensitized rabbit ERYTHROCYTES (AP_{50}) are incubated with dilutions of patient serum. ANTIBODY-sensitized sheep ERYTHROCYTES activate the CLASSICAL PATHWAY, non-sensitized rabbit ERYTHROCYTES the ALTERNATIVE PATHWAY. The activity of the serum sample is then expressed in units representing the reciprocal of the dilution of serum that lyses 50% of the ERYTHROCYTES. The CH_{50} assay measures the overall activity of classical and common pathways, the AP_{50} assay that of the alternative and the common pathways. These HEMOLYTIC ASSAYS were the first to be applied in clinical studies. Decreased hemolytic activity of sera may occur during activation of complement in patients, because activated complement factors are cleared from the circulation more rapidly than non-activated (native) complement proteins. However, during an ongoing ACUTE-PHASE REACTION, a decrease in complement protein levels may be masked by increased synthesis. Immunochemical determination of individual complement proteins, for example by nephelometry, has now largely replaced $CH_{50}$and AP_{50} determinations, the more so since the pattern of the relative decreases of complement proteins may provide important diagnostic and prognostic information. Nowadays, CH_{50} and AP_{50} determinations should only be used to screen for the presence of genetic deficiencies. Deficiencies of the CLASSICAL PATHWAY will yield decreased activity in the CH_{50} assay, those of the ALTERNATIVE PATHWAY lead to decreased AP_{50} activity. Deficiencies of C3–C9 will yield decreased activity in either assay.

Next to HEMOLYTIC ASSAYS, novel ELISA-based assays have now become available to assess complement function of all three activation pathways. These assays make use of specific ligands fixed to a solid phase for each complement activation pathway (i.e., IgM for the CLASSICAL PATHWAY, mannan for the LECTIN PATHWAY and LPS for the ALTERNATIVE PATHWAY). Completion of the activation pathways is detected with a monoclonal ANTIBODY against a NEO-EPITOPE on C9 [21].

Activation of complement in patients can best be assessed by measuring levels of specific complement activation products, such as levels of the ANAPHYLATOXINS (in particular C3a), C3b, C4b or circulating C5b–C9 complexes. The availability of MONOCLONAL ANTIBODIES specifically reacting with NEO-EPITOPES exposed on activation products and not cross-reacting with the native protein has greatly facilitated the development of specific, sensitive and reproducible immunoassays for these activation products, which are now frequently used in clinical practice.

Clinical relevance

The COMPLEMENT SYSTEM plays a pivotal role in human disease. On the one hand, complement activation has many functions in immunity and deficiencies within the COMPLEMENT SYSTEM may lead to increased susceptibility to invasive bacterial infections or development of AUTOIMMUNE DISEASES. On the other hand, undesired or excessive complement activation is a major cause of tissue injury in many pathological conditions.

Deficiencies in the early components of the CLASSICAL PATHWAY (C1q, C4 and C2) are associated with an increased risk for the development of AUTOIMMUNE DISEASE, especially SYSTEMIC LUPUS ERYTHEMATOSUS (SLE).

Recently, it has become clear that CLEARANCE of apoptotic cells is an important function of the CLASSICAL PATHWAY of complement. Deficiencies in this pathway may therefore lead to prolonged exposure of the specific IMMUNE SYSTEM to apoptotic cells and debris, leading to auto-ANTIBODY responses. Indeed most, if not all, auto-ANTIBODIES in patients with SLE are directed against EPITOPES exposed by apoptotic cells. This is known as the "waste disposal hypothesis" [23, 24].

Deficiencies of C3 are associated with recurrent infections by pyogenic micro-organisms, because of lack of OPSONIZATION and inability to use the membrane attack pathway. Finally, deficiencies of C5–C8 may lead to an increased risk for *Neisseria* infections. Surprisingly, C9 deficiencies are not associated with an increased risk for infections. Hence, OPSONIZATION of micro-organisms by C3 is apparently essential for defense against pyogenic bacteria, whereas the formation of complement pores contributes to defense against *Neisseria*. MBL levels may vary widely in the normal population and 15–20% of the people in the Western world having strongly decreased levels of functional MBL. A number of studies have shown that individuals with low levels of MBL have an increased risk for infections, particularly when immunity is already compromised, e.g., in infants, and in patients with cystic fibrosis and after chemotherapy or transplantation [25].

Activation of complement is considered to play an important role in the pathogenesis of a number of inflammatory disorders, including sepsis and septic SHOCK, toxicity induced by the *in vivo* administration of CYTOKINES or MONOCLONAL ANTIBODIES, immune complex diseases, such as RHEUMATOID ARTHRITIS, SLE and vasculitis, multiple trauma, ischemia-reperfusion injuries and myocardial infarction. The pathogenic role of complement activation in these conditions is probably related somehow to the biological effects of its activation products (Tab. 3). Inhibition of complement activation may therefore be beneficial in these conditions, which is substantiated by observations in animal models. The availability of clinically applicable complement inhibitors may help in the treatment of these diseases. Recently, a C5 blocking monoclonal ANTIBODY (eculizumab) has been approved for treatment of the autoimmune disorder, paroxysmal nocturnal hemoglobinuria (PNH), illustrating the critical role of C5 in this disease [22]. Currently, clinical trials are being conducted to evaluate this novel complement inhibiting drug in other diseases.

The first complement inhibitor that became available for clinical use was C1-inh, a major inhibitor of the CLASSICAL PATHWAY. It has been used largely due to the fact that a heterozygous deficiency state of this inhibitor is associated with the clinical picture of hereditary angio-edema (HAE). This disease sometimes leads to the development of life-threatening edema of the glottis, which must be treated with intubation and the intravenous administration of purified C1-inh. The pathogenesis of angio-edema attacks associated with low C1-inh levels is not completely clear but probably involves the generation of C2 peptide and BRADYKININ. The generation of these peptides results from the unopposed action of activated C1, activated coagulation factor XII and kallikrein of the contact system (C1-inh is the main inhibitor of this system as well). Importantly, low levels of functional C1-inh may be caused by a genetic defect but may also be acquired. Acquired C1-inhibitor deficiency is often associated with the presence of auto-ANTIBODIES against C1-inh, which cause an accelerated consumption of C1-inh. These ANTIBODIES are usually produced by a malignant B cell CLONE. HAE can be treated by attenuated androgens such as Danazol, anti-fibrinolytic agents or intravenously administered C1-inh.

Summary

As indicated in this chapter, the three branches of the INNATE IMMUNE SYSTEM co-operate to protect against PATHOGENIC MICRO-ORGANISMS and to remove infected, dysregulated, damaged or outdated cells. PHAGOCYTES act by migrating to infected areas and by ingesting and killing micro-organisms. NK cells induce APOPTOSIS in virus-infected or tumor cells. MACROPHAGES remove apoptotic and aged cells. Finally, the COMPLEMENT SYSTEM helps PHAGOCYTES to find and ingest micro-organisms but also leads to direct lysis of microbes. Each of these systems is potentially dangerous to the host as well. Therefore, a very tight regulation of their activities exists to protect the host tissue against damage to innocent bystander cells

and against excessive and long-lasting activation of these systems. Therapeutic intervention in the case of deficiencies or dysregulation is limited, but the possibilities may increase as we gain more insight into the basic principles.

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Inflammatory mediators and intracellular signalling

Richard Korbut and Tomasz J. Guzik

Introduction

INFLAMMATION is a protective response of the macro-organism to injury caused by trauma, noxious chemicals or microbiological toxins. This response is intended to inactivate or destroy invading organisms, remove irritants, and set the stage for tissue repair. The inflammatory response consists of immunological and non-immunological reactions. The latter are triggered by the release from the injured tissues and migrating cells of lipid-derived autacoids, such as EICOSANOIDS or "PLATELET-ACTIVATING FACTOR" (PAF), large peptides, such as INTERLEUKIN-1, small peptides, such as BRADYKININ, and amines, such as HISTAMINE or 5-hydroxytryptamine (5-HT). These constitute the chemical network of the inflammatory response and result in clinical and pathological manifestations of INFLAMMATION (Tab. 1). The concept of the inflammatory response was introduced over 2000 years ago with its description by Cornelius Celsus as "*rubor et tumor cum calore et dolore*" (redness and swelling with heat and pain). Centuries later, in the 19th century this definition was extended by Rudolph Virchow to include loss of function ("*functio laesa*"). It was Virchow and his pupils, including J. Cohnheim, who explained the scientific basis for Celsus' description of INFLAMMATION. They found that the redness and heat reflected increased blood flow, that the swelling was related to the exudation of fluid and to the accumulation of cells, while pain follows [1]. The first understanding of the mechanism of INFLAMMATION was introduced by Elie Metchnikoff, who concluded in his book "Comparative Pathology of INFLAMMATION" published in 1893, that "…INFLAMMATION is a local reaction, often beneficial, of living tissue against an irritant substance" [2]. This definition still stands today. For the first time, he observed that this reaction is mainly produced by phagocytic activity of the mesodermic cells, and that it includes "the chemical action of the blood plasma and tissue fluids…", thus introducing the concept of mediators of INFLAMMATION [2]. Numerous further studies since then have identified the roles of individual mediators in INFLAMMATION, and we are beginning to understand the genetic and molecular aspects of the genesis of the inflammatory process. Inflammatory mediators include a plethora of cell-derived molecules [e.g. CHEMOKINES, CYTOKINES, ANTIMICROBIAL PEPTIDES, and reactive oxygen (ROS) and nitrogen species] and of activated biochemical cascades originating in the vascular compartment [e.g. ROS, NITRIC OXIDE (NO), complement, coagulation, and fibrinolytic systems].

Eicosanoids

Arachidonic acid (AA) metabolites are formed rapidly from lipids of the cellular membrane, following activation of cells by numerous chemical and physical stimuli. They exert their effects locally (autacoids), affecting virtually every step of INFLAMMATION [3]. EICOSANOIDS encompass cyclic prostanoid structures, i.e. PROSTAGLANDINS (PGs), PROSTACYCLIN (PGI_2), THROMBOXANE A_2 (TXA_2), and also straight chain LEUKOTRIENE structures (LTs), i.e. chemotactic LTB_4 and pro-inflammatory peptidolipids (LTC_4, LTD_4, LTE_4) (Fig. 1). Subsequently, a new group of molecules was added to the family of EICOSANOIDS, namely LIPOXINS (LXA_4 and LXB_4), which are products of platelet 12-LIPOXYGENASE metabolism of neutrophil LTA_4 (TRANSCELLULAR BIOSYNTHESIS). EICOSANOIDS are synthesised by the cyclooxygenation (prostanoids) or lipoxygenation (LEUKOTRIENES) of a 20-carbon ω-6 polyunsaturated fatty acid (PUFA) – 5,8,11,14-eicosa-

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Table 1. Symptoms of inflammation induced by inflammatory mediators

Symptom	Mediators
Vascular permeability	Vasoactive amines Bradykinin Leukotrienes C_4, D_4, E_4 PAF Complement (C3a and C5a) Substance P Nitric oxide
Vasodilatation	Nitric oxide PGI_2, PGE_1, PGE_2, PGD_2 Hydrogen peroxide
Vasoconstriction	Thromboxane A_2 Leukotrienes C_4, D_4, E_4 Superoxide
Chemotaxis and leukocyte adhesion	Chemokines LTB_4, HETE, lipoxins Complement (C5a) Bacterial antigens
Pain	Bradykinin Prostaglandins
Fever	IL-1, TNF, IL-6 Prostaglandins
Tissue and endothelial damage	Reactive oxygen species Nitric oxide Lysosomal enzymes

tetraenoic acid (AA, arachidonic acid) (Fig. 1). AA is an important structural constituent of cellular phospholipids, and first must be liberated by acylhydrolases – directly by phospholipase A_2 (PLA_2) or indirectly by PLC before it becomes the SUBSTRATE for the synthesis of EICOSANOIDS.

Prostanoids

Prostanoids are produced by the CYCLOOXYGENASE PATHWAY. PROSTAGLANDIN H synthase (PGHS) is a dimeric complex that contains CYCLOOXYGENASE (COX) and peroxidase (Px). The COX cyclises the AA to an unstable cyclic 15-hydroperoxy PROSTAGLANDIN endoperoxide (PGG_2), while the Px converts the 15-hydroperoxy to a 15-hydroxy group, in this way yielding PGH_2. Eventually, the end-product of PGHS (the complex that contains either constitutive COX-1, inducible COX-2 or the COX-3) is an unstable cyclic PROSTAGLANDIN endoperoxide (PGH_2), which in various types of cells is converted by corresponding isomerases or synthases to the stable prostanoids: PGD_2, PGE_2, $PGF_{2\alpha}$, and unstable prostanoids, i.e. PGI_2 or TXA_2. Special biological significance has been ascribed to PGI_2 synthase in vascular endothelial cells and TXA_2 synthase in blood platelets. The transcellular metabolism providing PGH_2 from activated platelets to endothelial cells is the main source of vascular PGI_2 [4]. The biological activity of stable prostanoids is terminated by catabolic enzymes, such as PROSTAGLANDIN 15-hydroxy dehydrogenase (15-PGDH), D^{13}-reductase or α and ω oxidases, which are present in high concentration in the lungs. These enzymes also break down inactive TXB_2 and 6-keto-$PGF_{1\alpha}$.

The role of individual COX enzymes in the development of INFLAMMATION remains unclear. The discovery of the inducible form, COX-2, led to the hypothesis that COX-1 is a constitutive enzyme responsible for the physiological activities of PROSTAGLANDINS while COX-2, which is expressed during INFLAMMATION, produces "bad" PROSTAGLANDINS, which generate pain and fever (see chapter C14). This hypothesis quickly turned out to be simplistic and both enzymes show their activities under both physiological and pathological conditions [5]. Moreover, COX-2 inhibitory drugs possess less analgesic properties than non-selective inhibitors. The picture became even more complicated in 2002 with the discovery of COX-3. This isoenzyme is not a separate genetic isoform (like COX-2), but a splice variant of COX-1. In fact, COX-1 mRNA gives rise to four different isoforms including classical COX-1, COX-3 (splice variant including intron 1) and two partially truncated, inactive PCOX-1a and 1b. COX-3 due to the presence of intron 1, which changes its conformational structure, shows significantly diminished activity (25%) [6]. It is expressed mainly in the human brain and the heart. It has been suggested that COX-3 is an isoform particularly involved in the mechanisms of pain and fever during INFLAMMATION.

Some suggestions exist that this isoform is inhibited by paracetamol, which could explain its analgesic actions (see chapter C14).

The biosynthesis of prostanoids is initiated by transductional mechanisms in an immediate response to the activation of various cell membrane RECEPTORS or to various physical and chemical stimuli. These lead to an increase in the cytoplasmic levels of calcium ions $[Ca^{2+}]_i$ and in this way they activate acyl hydrolases, which thereby release free AA for metabolism by the PGHS. Alternatively, these enzymes can be induced by delayed transcriptional mechanisms, which are usually activated by CYTOKINES or bacterial toxins. The spectrum of prostanoids produced by individual tissues depends on the local expression of individual enzymes. For example, vascular endothelium possesses PROSTACYCLIN synthase and COX-2, but lacks THROMBOXANE synthase, present in turn in the platelets. Accordingly, the major prostanoid released by endothelium is PGI_2, while platelets produce TXA_2.

Prostanoids regulate vascular tone and permeability in the development of INFLAMMATION. They (TX) also induce platelet aggregation and thrombus formation. PROSTAGLANDINS (in particular PGE_2) are also involved in the pathogenesis of the pain and fever that accompany INFLAMMATION.

Most actions of prostanoids appear to be brought about by activation of the cell surface RECEPTORS that are coupled by G proteins to either adenylate cyclase (changes in intracellular c-AMP levels) or PLC (changes in triphosphoinositol – IP_3 and diacylglycerol – DAG levels) [7]. The diversity of the effects of prostanoids is explained by the existence of a number of distinct RECEPTORS [8]. The RECEPTORS have been divided into five main types, designated DP (PGD), FP (PGF), IP (PGI_2), TP (TXA_2), and EP (PGE). The EP RECEPTORS are subdivided further into EP_1 (smooth muscle contraction), EP_2 (smooth muscle relaxation), EP_3 and EP_4, on the basis of physiological and molecular cloning information. Subtype-selective RECEPTOR antagonists are under development. Only one gene for TP RECEPTORS has been identified, but multiple splice variants exist. PGI_2 binds to IP RECEPTORS and activates adenylate cyclase. PGD_2 interacts with a distinct DP RECEPTOR that also stimulates adenylate cyclase. PGE_1 acts through IP RECEPTORS, PGE_2 activates EP RECEPTORS but it may also act on IP and DP RECEPTORS.

While most PROSTAGLANDINS participate in the pathomechanism of INFLAMMATION, a more recently discovered member of this family – the 15-deoxy-Δ-12,14-PROSTAGLANDIN J2 (15d-PGJ2) – is the dehydration end product of the PGD_2, and differs from other PROSTAGLANDINS in several respects [9]. 15d-PGJ2 has been shown to act *via* PGD_2 RECEPTORS (DP1 and DP2), and through interaction with intracellular targets. In particular, 15d-PGJ2 is recognised as the endogenous LIGAND for the intranuclear RECEPTOR PPARγ. This property is responsible for many of the anti-inflammatory functions of 15d-PGJ2.

Products of the lipoxygenation of arachidonic acid

AA can be metabolised to straight chain products by lipoxygenases (LOXs), which are a family of cytosolic enzymes that catalyse oxygenation of all polyenic fatty acids with two *cis* double bonds that are separated by the methylene group to corresponding lipid hydroperoxides [10] (Fig. 1). As in the case of AA, these hydroperoxides are called hydroperoxyeicosatetraenoic acids (HPETEs). Different LOX enzymes vary in their SPECIFICITY for inserting the hydroperoxy group and tissues differ in the LOXs that they contain. Platelets contain only 12-LOX and synthesise 12-HPETE, whereas LEUKOCYTES contain both 5-LOX and 12-LOX producing both 5-HPETE and 12-HPETE. HPETEs are unstable intermediates, analogous to PGG_2 or PGH_2, and are further transformed by peroxidases or non-enzymatically to their corresponding hydroxy fatty acids (HETEs). 12-HPETE can also undergo catalysed molecular rearrangement to epoxy-hydroxyeicosatrienoic acids called hepoxillins. 15-HPETE may also be converted by lipoxygenation of LTA_4 to the trihydroxylated derivatives, the LIPOXINS (Fig. 1).

Leukotrienes

In activated LEUKOCYTES, an increase in $[Ca^{2+}]_i$ binds 5-LOX to five-LIPOXYGENASE-activating-protein (FLAP),

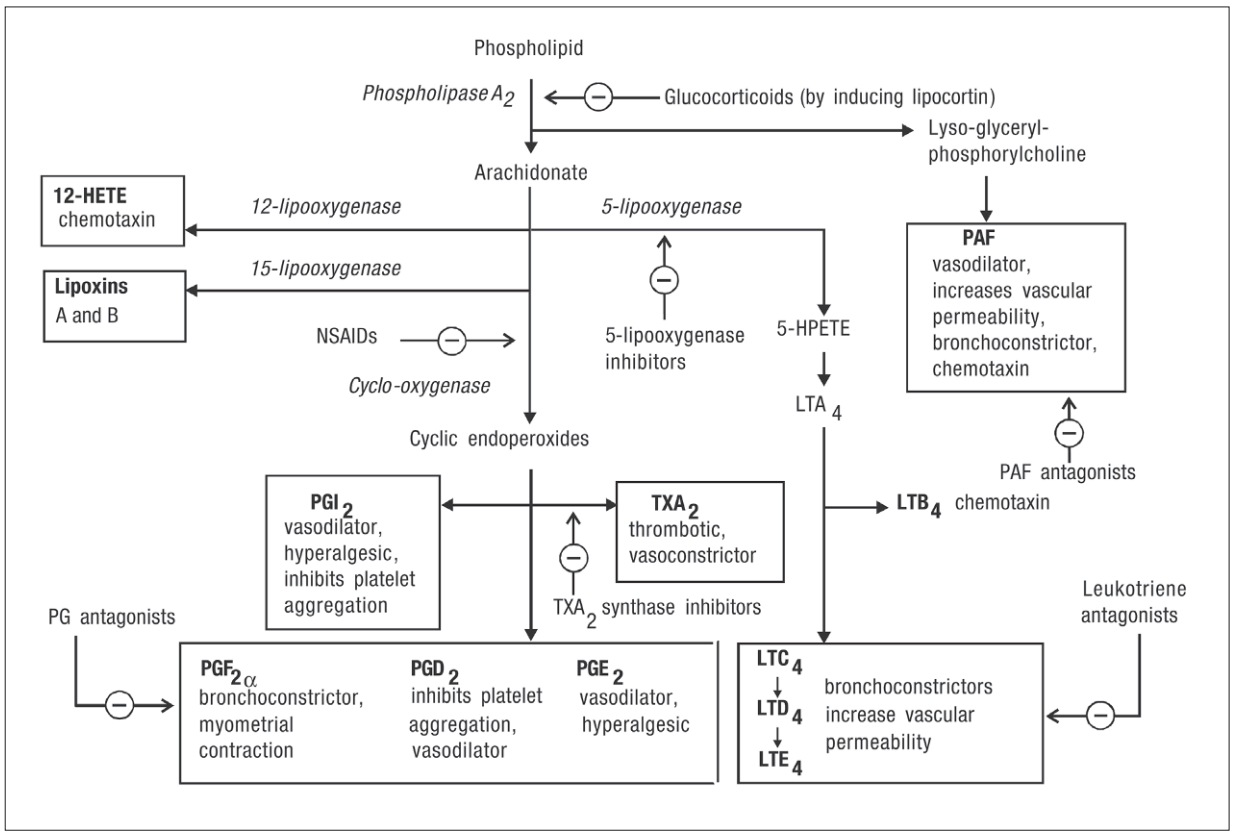

FIGURE 1
Mediators derived from phospholipids and their actions, the sites of action of anti-inflammatory drugs.

and this complex converts AA to 5-HPETE, which in turn is the SUBSTRATE for LTA_4 synthase. In the course of transcellular metabolism between LEUKOCYTES and blood cells or endothelial cells, unstable LTA_4 is converted by corresponding enzymes to stable chemotactic LTB_4 or to CYTOTOXIC cysteinyl-containing LEUKOTRIENES C_4, D_4, E_4 and F_4 (also referred to as sulphidopeptide LEUKOTRIENES or peptidolipids) [11] (Fig. 1). Note that the transcellular metabolism of AA can bring about either "protection", as is the case during the platelet/endothelium transfer of PGH_2 to make cytoprotective PGI_2 [1], or "damage", as in the case of the leukocyte/endothelium transfer of LTA_4 to make CYTOTOXIC LTC_4 [6].

Consecutive splicing of amino acids from the glutathione moiety of LTC_4 occurs in the lungs, kidney, and liver. LTE_4 is already substantially deprived of most of the biological activities of LTC_4 and LTD_4. Also LTC_4 may be inactivated by oxidation of its cysteinyl sulphur atom to a sulphoxide group. The principal route of inactivation of LTB_4 is by ω-oxidation. LTC_4 and LTD_4, comprise an important endogenous bronchoconstrictor, previously known as the "slow-reacting substance of ANAPHYLAXIS" (SRS-A) [12].

Three distinct RECEPTORS have been identified for LTs (LTB_4, LTC_4 and LTD_4/LTE_4). Stimulation of all of them appears to activate PLC. LTB_4, acting on specific RECEPTORS, causes ADHERENCE, CHEMOTAXIS and activation of POLYMORPHONUCLEAR LEUKOCYTES and MONOCYTES, as well as promoting cytokine production in MACROPHAGES and LYMPHOCYTES. Its potency

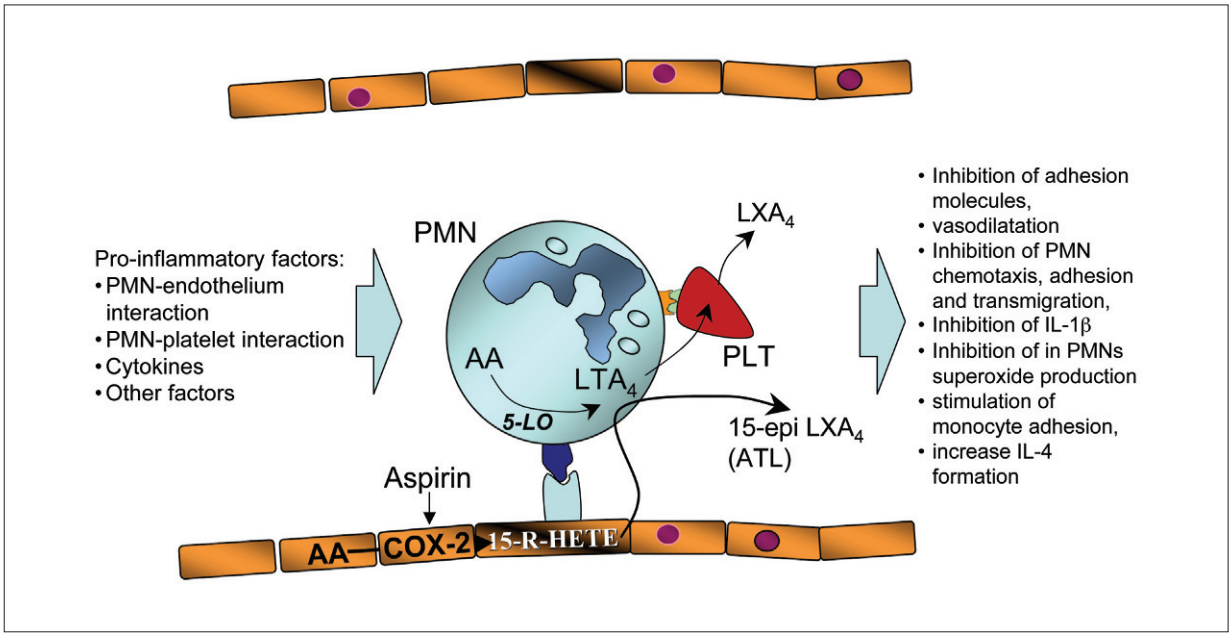

FIGURE 2
Transcellular synthesis of lipoxins and their actions.

is comparable with that of various chemotactic peptides and PAF. At higher concentrations, LTB_4 stimulates the aggregation of PMNs and promotes DEGRANULATION and the generation of superoxide. It promotes adhesion of NEUTROPHILS to vascular endothelium and their transendothelial migration [13]. The cysteinyl-LTs are strongly CYTOTOXIC, and cause bronchoconstriction and vasodilatation in most vessels except the coronary vascular bed.

Lipoxins (lipoxygenase interaction products)

LIPOXINS are formed by a sequential transcellular metabolism of AA by 15- and 5-, or by 5- and 12-lipooxygenases [14]. The cellular context is critical for the synthesis of LIPOXINS (Fig. 2). LIPOXINS have several anti-inflammatory properties as well as concomitant pro-inflammatory actions. LIPOXINS inhibit the ADHESION MOLECULE expression on endothelium, cause vasodilatation and attenuate LTC_4-induced vasoconstriction by antagonism of $cysLT_1$ RECEPTOR. They also inhibit CHEMOTAXIS, adhesion and transmigration, and IL-1β and superoxide production of POLYMORPHONUCLEAR LEUKOCYTES. On the other hand, LIPOXINS stimulate monocyte adhesion and increase IL-4 formation [14, 15]. There is an inverse relationship between the amount of lipoxin and LEUKOTRIENE production, which may indicate that LIPOXINS may be "endogenous regulators of LEUKOTRIENE actions". High-AFFINITY G protein-coupled lipoxin RECEPTORS (ALXR) have been identified on numerous cells, including MONOCYTES, PMNs, fibroblasts and endothelial and epithelial cells. RECEPTOR expression may be up-regulated by interferon (IFN)-γ, IL-13 or even IL-1β. Activation of this RECEPTOR modulates phosphatidylinositide 3-kinase (PI3-kinase) activity. LIPOXINS may also competitively bind and block the $cys\text{-}LT_1$ RECEPTOR. There are also suggestions that LIPOXINS may also bind within the cell to LIGAND-activated transcription factors, therefore regulating GENE EXPRESSION in the nucleus.

A separate group of LIPOXINS was termed aspirin-triggered LIPOXINS (ATLs), as their synthesis is the result

of acetylation of COX-2, which inhibits endothelial cell prostanoid formation and promotes synthesis of 15(*R*) HETE. These are then converted in PMNs to 15*R*-enantiomeres: 15-epi LXA_4 or 15-epi-LXB_4. ATLs share many actions of LIPOXINS, albeit with much greater potency [16]. Due to their anti-inflammatory properties, lipoxin analogues may find an important place in the treatment of INFLAMMATION [14, 15].

Other pathways of arachidonic acid metabolism

AA can be also metabolised by an NADPH-dependent cytochrome P450-mediated monooxygenase pathway (MOX). The resulting 19-HETE, 20-HETE and a number of epoxyeicosatrienoic and dihydroxyeicosatrienoic acid isomers show vascular, endocrine, renal, and ocular effects, the physiological importance of which remains to be elucidated [17].

Recently, a non-enzymatic, FREE RADICAL-mediated oxidation of AA, while still embedded in phospholipids, has been discovered. Subsequently, acyl hydrolases give rise to a novel series of regioisomers of ISOPROSTANES. Formed non-enzymatically, ISOPROSTANES lack the stereospecificity of prostanoids. Highly toxic ISOPROSTANES might contribute to the pathophysiology of inflammatory responses which are insensitive to currently available steroidal and non-steroidal anti-inflammatory drugs. The most thoroughly investigated regioisomer of ISOPROSTANES is 8-epi-$PGF_{2\alpha}$. It has a potent vasoconstrictor action, which is mediated by vascular TXA_2/PGH_2 RECEPTORS.

Actions and clinical uses of eicosanoids

EICOSANOIDS produce a vast array of biological effects. TXA_2, $PGF_{2\alpha}$ and LTs represent CYTOTOXIC, pro-inflammatory mediators. TXA_2 is strongly thrombogenic through aggregation of blood platelets. LTC_4 injures blood vessels and bronchi subsequent to activation of LEUKOCYTES. On a molecular level, their CYTOTOXICITY is frequently mediated by stimulation of PLC or inactivation of adenylate cyclase. Cytoprotective, but not necessarily anti-inflammatory actions are mediated by PGE_2 and PGI_2. They are both naturally occurring vasodilators. PGI_2 is the most comprehensive anti-platelet agent and is responsible for the thromboresistance of the vascular wall. PGE_2 through a similar adenylate cyclase-dependent mechanism inhibits the activation of LEUKOCYTES. PGE_2 is also responsible for protection of the gastric mucosa. PGE_2 and $PGF_{2\alpha}$ may play a physiological role in labour and are sometimes used clinically as abortifacients. Locally generated PGE_2 and PGI_2 modulate vascular tone and the importance of their vascular actions is emphasised by the participation of PGE_2 and PGI_2 in the hypotension associated with septic SHOCK. These PROSTAGLANDINS have also been implicated in the maintenance of patency of the ductus arteriosus. Various PROSTAGLANDINS and LEUKOTRIENES are prominent components released when sensitised lung tissue is challenged by the appropriate antigen. While both bronchodilator (PGE_2) and bronchoconstrictor ($PGF_{2\alpha}$, TXA_2, LTC_4) substances are released, responses to the peptidoleukotrienes probably dominate during allergic constriction of the airway. The relatively slow metabolism of the LEUKOTRIENES in lung tissue contributes to the long-lasting bronchoconstriction that follows challenge with antigen and may be a factor in the high bronchial tone that is observed in asthmatics in periods between attacks. PROSTAGLANDINS and LEUKOTRIENES contribute importantly to the genesis of the signs and symptoms of INFLAMMATION. The peptidoleukotrienes have effects on vascular permeability, while LTB_4 is a powerful chemoattractant for POLYMORPHONUCLEAR LEUKOCYTES and can promote exudation of plasma by mobilising the source of additional inflammatory mediators. PROSTAGLANDINS do not appear to have direct effects on vascular permeability. However, PGE_2 and PGI_2 markedly enhance oedema formation and leukocyte infiltration by promoting blood flow in the inflamed region. PGEs inhibit the participation of LYMPHOCYTES in delayed HYPERSENSITIVITY reactions. BRADYKININ and the CYTOKINES (such as TNF-α, IL-1, IL-8) appear to liberate PROSTAGLANDINS and probably other mediators that promote hyperalgesia (decreased pain threshold) and the pain of INFLAMMATION. Large doses of PGE_2 or $PGF_{2\alpha}$ given to women by intramuscular or subcutaneous injection to induce abortion, cause intense local pain. PROSTAGLANDINS also can cause headache and vascular pain when infused intravenously. The capacity of PROSTAGLANDINS to sensitise pain RECEPTORS to mechanical

and chemical stimulation appears to result from a lowering of the threshold of the polymodal nociceptors of C fibres. Hyperalgesia also is produced by LTB_4. PGE_2 when infused into the cerebral ventricles or when injected into the hypothalamus produces fever. The mechanism of fever involves the enhanced formation of CYTOKINES that increase the synthesis of PGE_2 in circumventricular organs in and near to the preoptic hypothalamic area, and PGE_2, *via* increases in c-AMP, triggers the hypothalamus to elevate body temperature by promoting increases in heat generation and decreases in heat loss.

SYNTHETIC PGE_1, acting through IP and EP RECEPTORS, is given by infusion to maintain the patency of the ductus arteriosus in infants with transposition of large vessels until surgical correction can be undertaken. PGI_2 (epoprostenol) is occasionally used to prevent platelet aggregation in dialysis machines through inhibition of the thrombocytopenic action of heparin [18]. PGI_2 is also used for the treatment of primary and secondary pulmonary hypertension [19]. Stable analogues of PGI_2 (e.g. iloprost) as well as of PGE_1 are used in selected patients with peripheral vascular disease [18]. The PGE_1 analogue, misoprostol is approved in the USA for the prevention of peptic ulcers, especially in patients who are required to take high doses of non-steroidal anti-inflammatory drugs (NSAID) for treatment of their arthritis.

Pharmacological interference with eicosanoid synthesis and actions

PLA_2 and COX are inhibited by drugs that are the mainstays in the treatment of INFLAMMATION. We discovered that glucocorticosteroids (hydrocortisone, dexamethasone) inhibit the generation of prostanoids *in vivo* through prevention of the release of AA from phospholipids [11]. This effect is mediated by intracellular steroid RECEPTORS, which, when activated, increase expression of lipocortins that inhibit phospholipases. Many other actions of glucocorticosteroids on AA metabolism are known, one of them being inhibition of COX-2 transcription. These problems are further discussed in chapter C14.

Aspirin selectively inhibits COX-1, explaining its inhibitory effect on the biosynthesis of TXA_2 in platelets (causing reduced thrombotic tendency), of PGI_2 in endothelial cells and of PGE_2 in gastric mucosa (leading to gastric damage) (see chapter C14). This action of aspirin is more pronounced than that on the biosynthesis of prostanoids at the site of INFLAMMATION, where inducible COX-2 is most active. Consequently, aspirin at low doses seems to be a better anti-thrombotic than anti-inflammatory drug. Aspirin irreversibly acetylates the active centre of COX-1. Unlike endothelial cells, platelets lack the machinery required for *de novo* synthesis of COX-1, and, accordingly, aspirin-induced inhibition of TXA_2 synthesis in platelets is essentially permanent (until new platelets are formed), in contrast to the easily reversible inhibition of PGI_2 synthesis in vascular endothelium. The net effect of aspirin is, therefore, a long-lasting anti-thrombotic action. Unfortunately, most NSAIDs are more effective inhibitors of COX-1 than of COX-2. Meloxicam was the first clinically available drug that is claimed to be a selective COX-2 inhibitor – an anti-inflammatory drug with few side effects on the gastrointestinal tract, which causes no bleeding. However, population studies have verified that, while protective for gastric mucosa, high doses of COX-2 selective inhibitors may induce cardiovascular (due to inhibition of endothelial COX-2) or renal side effects [20]. NSAIDs usually are classified as mild analgesics and they are particularly effective in settings in which INFLAMMATION has caused sensitisation of pain RECEPTORS to normally painless mechanical or chemical stimuli. NSAIDs do not inhibit fever caused by direct administration of PROSTAGLANDINS, but they do inhibit fever caused by agents that enhance the synthesis of IL-1 and other CYTOKINES, which presumably cause fever at least in part by inducing the endogenous synthesis of PROSTAGLANDINS.

Platelet-activating factor

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a specialised phospholipid with an alkyl group (12-18C) attached by an ether bond at position 1 of glycerol and acetylated at position 2. PAF is not stored in cells but it is synthesised from 1-*O*-alkyl-2-

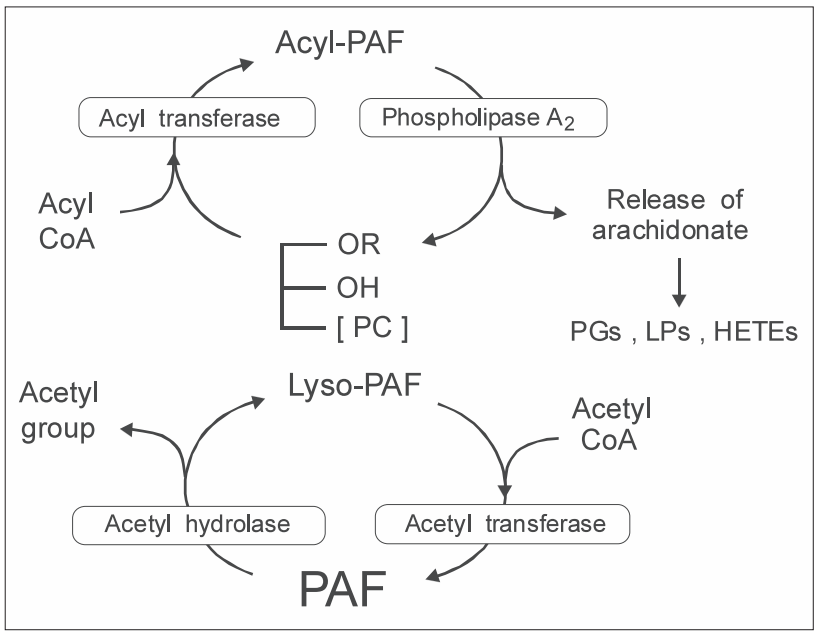

FIGURE 3
The synthesis and metabolism of platelet-activating factor (PAF)

acyl-glycerophosphocholine as required (Fig.3) [21]. Initially, PLA_2 converts the precursor to the inactive 1-*O*-alkyl-2-lysoglycerophosphocholine (lyso-PAF) with concomitant release of AA. Incidentally, in GRANULOCYTES, AA produced in this way represents a major source for the synthesis of PGs and LTA_4. In a second step, lyso-PAF is acetylated by acetyl coenzyme A in a reaction catalysed by lyso-PAF acetyltransferase. This is the rate-limiting step. The synthesis of PAF in different cells is stimulated during antigen-ANTIBODY reactions or by chemotactic peptides (e.g. f-MLP), CYTOKINES, thrombin, collagen, and autacoids. PAF can also stimulate its own formation. Both PLA_2 and lyso-PAF acetyltransferase are calcium-dependent enzymes, and PAF synthesis is regulated by the availability of Ca^{2+}. The anti-inflammatory action of glucocorticosteroids is at least partially dependent on inhibition of the synthesis of PAF by virtue of the inhibitory effect of lipocortin on the activity of PLA_2.

Inactivation of PAF also occurs in two steps (Fig. 3) [21]. Initially, the acetyl group of PAF is removed by PAF acetylhydrolase to form lyso-PAF, this enzyme is present in both cells and plasma. Lyso-PAF is then converted to a 1-*O*-alkyl-2-acyl-glycerophosphocholine by an acyltransferase. This latter step is inhibited by Ca^{2+}.

PAF is synthesised by platelets, NEUTROPHILS, MONOCYTES, BASOPHILS and MAST CELLS, EOSINOPHILS, renal mesangial cells, renal medullary cells, and vascular endothelial cells. In most instances, stimulation of the synthesis of PAF results in the release of PAF and lyso-PAF from the cell. However, in some cells (e.g. endothelial cells) PAF is not released and appears to exert its effects intracellularly.

PAF exerts its actions by stimulating a single G protein-coupled, cell-surface RECEPTOR [22]. High-AFFINITY binding sites have been detected in the plasma membranes of a number of cell types. Stimulation of these RECEPTORS triggers activation of phospholipases C, D, and A_2, and mobilisation of $[Ca^{2+}]_i$. Massive direct and indirect release of AA occurs with its subsequent conversion to PGs, TXA_2, or LTs. EICOSANOIDS seem to function as extracellular representatives of the PAF message. As its name suggests, PAF unmasks fibrinogen RECEPTORS on platelets, leading directly to platelet aggregation. In endothelial cells, the synthesis of PAF may be stimulated by a variety of factors, but here PAF is not released extracellularly. Accumulation of PAF intracellularly is

associated with the adhesion of NEUTROPHILS to the surface of the endothelial cells and their DIAPEDESIS, apparently because it promotes the expression or exposure of surface proteins that recognise and bind NEUTROPHILS. Activated endothelial cells play a key role in "targeting" circulating cells to inflammatory sites. Expression of the various ADHESION MOLECULES varies among different cell types involved in the inflammatory response. For example, expression of E-SELECTIN is restricted primarily to endothelial cells and is enhanced at sites of INFLAMMATION. P-selectin is expressed predominantly on platelets and on endothelial cells. L-selectin is expressed on LEUKOCYTES and is shed when these cells are activated. Cell adhesion appears to occur by recognition of cell surface glycoprotein and carbohydrates on circulating cells by ADHESION MOLECULES of which expression has been enhanced on resident cells. Endothelial activation results in adhesion of LEUKOCYTES by their interaction with newly expressed L-selectin and P-selectin, whereas endothelial-expressed E-SELECTIN interacts with glycoproteins on the leukocyte surface, and endothelial ICAM-1 interacts with leukocyte INTEGRINS.

PAF also very strongly increases vascular permeability. As with substances such as HISTAMINE and BRADYKININ, the increase in permeability is due to contraction of venular endothelial cells, but PAF is 1000–10 000 times more potent than HISTAMINE or BRADYKININ.

Intradermal injection of PAF duplicates many of the signs and symptoms of INFLAMMATION, including vasodilatation, increased vascular permeability, hyperalgesia, oedema, and infiltration of NEUTROPHILS. Inhaled PAF induces bronchoconstriction, promotes local oedema, accumulation of EOSINOPHILS and stimulates secretion of mucus. In anaphylactic SHOCK, the plasma concentration of PAF is high and the administration of PAF reproduces many of the signs and symptoms of experimental anaphylactic SHOCK. PAF RECEPTOR antagonists prevent the development of pulmonary hypertension in experimental septic SHOCK. Despite the broad implications of these experimental observations, the clinical effects of PAF antagonists in the treatment of bronchial ASTHMA, septic SHOCK and other inflammatory responses have been rather modest.

PAF RECEPTOR antagonists include PAF structural analogues, NATURAL products (e.g. ginkgoloids from *Ginkgo biloba*), and interestingly, triazolobenzodiazepines (e.g. triazolam). The development of PAF RECEPTOR antagonists is currently at an early stage of clinical development, still leaving the hope that such antagonists may find future therapeutic application in INFLAMMATION and sepsis.

Innate immune signalling receptors

Several families of innate immune signalling RECEPTORS are currently known. Their functionality and subcellular location varies. These RECEPTORS include the transmembrane TOLL-LIKE RECEPTORS (TLR) and C-type LECTIN RECEPTORS, while other RECEPTORS are located in the cytosol, including the retinoic acid–inducible gene-I-like helicases (RLRs) and the nucleotide-binding domain leucine-rich repeat-containing RECEPTORS (NLRs). The common property and function of these RECEPTORS is to detect a broad variety of molecular entities including lipids, nucleic acids, proteins, and their combinations. It is most likely that these innate immune RECEPTORS evolved to recognise specific molecules associated with microbial invasion and thus are designed to orchestrate antimicrobial defence – virtually all of these RECEPTORS can also detect molecular changes that occur during tissue damage during virtually every kind of INFLAMMATION. Moreover, their importance may be emphasised by the fact that they are located at the beginning of the inflammatory cascade.

Toll-like receptors

The TOLL-LIKE RECEPTORS (TLR1–10) are a part of the innate immune defence, recognising conserved PATHOGEN-ASSOCIATED MOLECULAR PATTERNS (PAMPs) on microorganisms [23, 24]. TLRs and their signalling pathways are present in mammals, fruit flies, and plants. Ten members of the TLR family have been identified in humans, and several of them appear to recognise specific microbial products, including LIPOPOLYSACCHARIDE, bacterial lipoproteins, PEPTIDOGLYCAN, bacterial DNA and viral RNA. Signals initiated by the interaction of

TLRs with specific microbial patterns direct the subsequent inflammatory response including mononuclear phagocytic cell cytokine production. Thus, TLR signalling represents a key component of the innate immune response to microbial infection [23]. Interestingly, recent data indicates that TLRs play an important role not only in the modulation of INNATE IMMUNITY, but also in the initiation of specific responses of ADAPTIVE IMMUNITY (Tab. 2) [24]. Moreover, T cells express certain types of TLRs during development and activation stages and they participate in the direct regulation of adaptive immune response, possibly as COSTIMULATORY MOLECULES. COSTIMULATION of $CD4^+$ EFFECTOR CELLS with anti-CD3 mAb and TLR-5 LIGAND flagellin enhances T cell proliferation and production of IL-2 levels equivalent to those achieved by COSTIMULATION with classical ANTIGEN-PRESENTING CELL (APC) involving CD28. Moreover, CpG-containing oligodeoxynucleotides (CpG-ODN) can costimulate primary T cells in the absence of APCs. Finally, TLR activation on APCs may direct the development of immune responses into the T regulatory or Th17 pathway. These mechanisms are further discussed in chapters A2 and A4.

Inflammasomes

INFLAMMASOMES are cytoplasmic protein complexes critical in the regulation of INFLAMMATION [25]. The term was first introduced in 2002 to describe a caspase-1-activating multimolecular complex consisting of caspase-1, caspase-5, APOPTOSIS-associated speck-like protein containing a caspase recruitment domain (ASC), and NLRP1 (an NLR family member).

NLRs are a family of 20 intracellular immune RECEPTORS characterised by the presence of leucine-rich repeats (LRRs) near the C terminus and a central nucleotide-binding and oligomerisation (NACHT) nucleotide-binding domain (NBD). The LRR domains of this family are thought to play a role in autoregulation, the recognition of PAMPs, and/or protein-protein interactions. The NBDs can bind ribonucleotides, possibly regulating self-oligomerisation. In spite of these similarities, different NLRs differ in their N-terminal domains. Most of these have an N-terminal pyrine domain (PYD) and are therefore called NLRP (NALP). Other NLRs have an N-terminal caspase recruitment domain (CARD), and

TABLE 2. TOLL LIKE RECEPTORS IN THE REGULATION OF ADAPTIVE IMMUNITY

Toll-like receptor	Recognised molecular pattern of microorganisms	Effect on T cell function	Classical costimulatory effects
TLR1/2	Triacyl lipopeptides	Inhibition or reversal of T regulatory cells suppressive function	
TLR2	Peptidoglycan	Increase of T regulatory cell suppressive function	x
TLR3	ssRNA (viral), dsRNA, respiratory syncytial virus	Expressed; unclear function	x
TLR4	Lipopolysaccharide	Increase of T regulatory cell suppressive function	x
TLR5	Flagellin		x
TLR6	Lipoteichoic acid, Zymosan, Diacyl lipopeptides		
TLR7	ssRNA (viral; inc influenza)	Inhibition or reversal of T regulatory cells suppressive function	
TLR8	ssRNA (viral)		
TLR9	dsDNA (HSV); CpG dinucleotide motifs, haemozoin		x

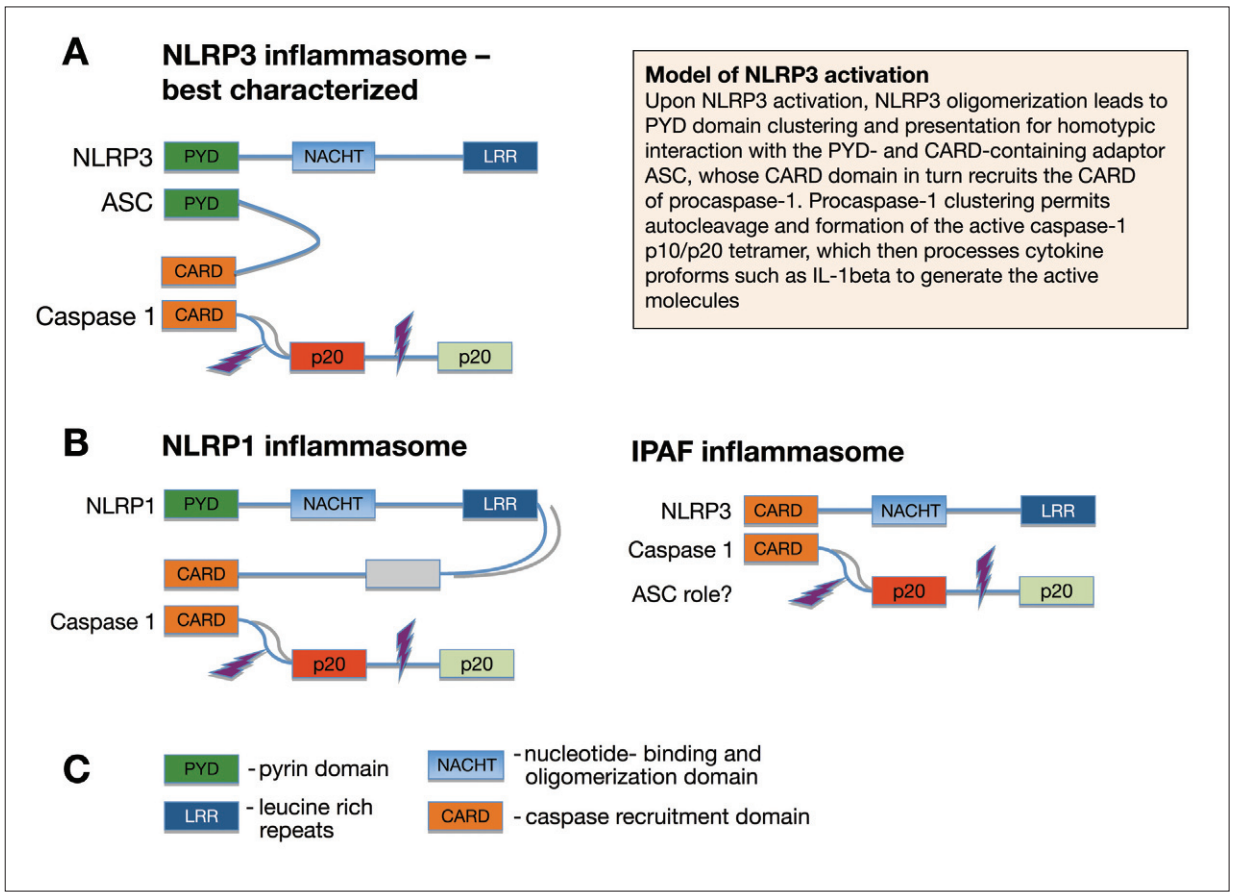

Figure 4. Simplified diagrams of examples of minimal inflammasomes: NLRP3 (A), NLRP1 and IPAF Inflammasomes (B). For simplicity, the unoligomerised inflammasome complexes are depicted. Main domains are briefly identified in (C). LRRs are believed to function in ligand sensing and autoregulation, whereas CARD and PYD domains mediate homotypic protein-protein interactions for downstream signalling. The NACHT domain, which is the only domain common to all NLR family members, enables activation of the signalling complex via ATP-dependent oligomerisation. Figure modified based on Cell *140, 821, 2010 , copyright licence by Elsevier no. 2440701030514.*

include NUCLEOTIDE-BINDING OLIGOMERISATION DOMAIN-containing-1 (NOD1, also known as NLRC1), NOD2 (NLRC2). CARD domain-containing-4 NLRs are also vital (NLRC4, also known as CARD12 or IPAF). Other NLR family members have an acidic TRANSACTIVATION DOMAIN or a baculoviral inhibitory repeat-like domain, such as the member of NLR family, APOPTOSIS inhibitory protein 5 (NAIP5).

The most important property of some NLR as discussed above is their ability to create INFLAMMASOMES. This involves particularly NLRP1, NLRP3, and NLRC4 which assemble multimolecular complexes in response to various activators, leading to the activation of inflammatory CASPASES. Through caspase-1 activation, the INFLAMMASOME controls the maturation of the CYTOKINES of the IL-1 family. The examples of minimal INFLAMMASOMES are demonstrated in the Figure 4.

For instance, an example is the NLRP3 INFLAMMASOME which can be activated by a myriad of

microbial factors, including Sendai virus, influenza A virus, adenoviruses, *Staphylococcus aureus*, *Listeria monocytogenes*, *E. coli*, *Mycobacterium marinum*, and *Neisseria gonorrhoeae* as well as the fungal *Candida albicans*. NLRP3 is activated by products of the above microbes, including MURAMYL DIPEPTIDE, bacterial RNA, LPS, Pam2CysK4, poly(I:C) as well as bacterial toxins such as nigericin or listeriolysin O (from *L. monocytogenes*), or α-toxin, and β- and γ-haemolysins (from *S. aureus*). In summary, activation of cytokine RECEPTORS or PATTERN RECOGNITION RECEPTORS such as TLRs leads to the induction of pro-IL-1β and NLRP3. In the next step, NLRP3 INFLAMMASOME assembly is triggered by low intracellular potassium levels (e.g. through formation of pores by bacterial toxins), which influences lysosomal stability and the binding of a putative LIGAND that is generated by proteolytic activity after lysosomal damage or by the action of ROS. The assembled NLRP3 INFLAMMASOME results in activation of caspase-1, which proteolytically activates IL-1β family CYTOKINES. The pro-inflammatory IL-1β family CYTOKINES can act on other cell types or act in a feed-forward loop. These mechanisms are excellently discussed in a recent review by Stutz et al. [25].

Cytokines

CYTOKINES are peptides produced by immune cells, which play key roles in regulating virtually all mechanisms of INFLAMMATION including INNATE IMMUNITY, antigen presentation, cellular differentiation, activation and recruitment as well as in repair processes. They are produced primarily by MACROPHAGES and LYMPHOCYTES, but also by other LEUKOCYTES, endothelial cells and fibroblasts. Substances considered to be CYTOKINES include IL-1–35, INTERFERONS (IFNs), tumour necrosis factors (TNF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, CHEMOKINES (which are discussed separately) and the colony-stimulating factors (CSFs). Major cytokine superfamilies are listed in Table 3 and the most important ones are discussed below. Further details are also given in chapter A5. The cytokine production profile in response to immune insult determines the nature of the immune response (cell-mediated, humoral, CYTOTOXIC or allergic) [26, 27].

Types of cytokines

Interleukin-1

IL-1 is the term given to a family of four CYTOKINES consisting of two active, agonists: IL-1α, IL-1β, an endogenous IL-1-RECEPTOR antagonist (IL-1ra) and the recently cloned cytokine IL-18, which is structurally related to IL-1. Both IL-1α, IL-1β and a related protein IL-18 are synthesised as a less active precursor. Their secretion in response to various stimuli (ANTIGENS, ENDOTOXIN, CYTOKINES or microorganisms) depends on

Table 3. Main cytokine families

Cytokine family	Cytokines
IL-1 superfamily	IL-1, IL-18, IL-33
IL-6 like cytokines	IL-6, IL-11, IL-27, IL-30, IL-31 (and oncostatins, cardiotrophin etc.)
IL-10 family	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26
Interferon type III (λ)	IL-28, IL-29
Common γ chain family	IL-2/15; IL-3; IL-4; IL-7; IL-9; IL-13; IL-21
IL-12 family	IL-12, IL-23, IL-27, IL-35
Other	IL-5, IL-8; IL14; IL-16; IL-17/25(A); IL-32; IL-34
IL-17 family	IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F
TNF ligand superfamily	TNF-α; 4-1BB ligand; B-cell activating factor; FAS ligand; TNF-β (lymphotoxin); OX40L; RANKL; TRAIL
Interferons	I (α) II (γ) III (IL-28; IL-29)

the cleavage of the pro-CYTOKINES to their active forms by IL-1 converting enzyme (ICE or caspase 1). IL-1α remains cell associated and is active mainly during cell-to-cell contact, while the soluble IL-1β is a form predominant in biological fluids. IL-1 is an important inflammatory mediator and it is believed to be implicated in several acute [e.g. systemic inflammatory response syndrome (SIRS) in sepsis] or chronic (e.g. RHEUMATOID ARTHRITIS) inflammatory diseases. IL-1 is also important in regulating adaptive immune responses.

One of the principal actions of IL-1 is activation of T LYMPHOCYTES and B cells by enhancing the production of IL-2 and expression of IL-2 RECEPTORS. In IL-1 knockout animals diminished immune responses or state of TOLERANCE is observed. In vascular endothelial cells, IL-1 increases the synthesis of leukocyte ADHESION MOLECULES (VCAM-1, ICAM-1 and E-SELECTIN), stimulates NO production, releases platelet-derived growth factor (PDGF), and activates PLA_2, thus inducing the synthesis of prostanoids and PAF. It stimulates fibroblasts to proliferate, to synthesise collagen and to generate collagenase. It regulates the systemic inflammatory response by stimulating synthesis of acute phase proteins (C-reactive protein, amyloid and complement), producing neutrophilia, and causing fever by altering a set-point of temperature in the hypothalamus. IL-1 also induces the generation of other CYTOKINES such as the IFNs, IL-3, IL-6, and, in BONE MARROW, the CSFs. It synergises with TNF-α in many of its actions, and its synthesis is stimulated by TNF-α. The therapeutic effects of GLUCOCORTICOIDS in RHEUMATOID ARTHRITIS and other chronic inflammatory and AUTOIMMUNE DISEASES may well involve inhibition of both IL-1 production and IL-1 activity. Production of IL-1ra alleviates potentially deleterious effects of IL-1 in the NATURAL course of the disease.

IL-18, although structurally close to the IL-1 family, exerts actions more related to IL-12. It was originally derived from liver, but is produced by numerous cell types (including lung, kidney and smooth muscle cells) apart from LYMPHOCYTES. In contrast to other CYTOKINES, IL-18 procytokine is constitutively expressed and therefore its activity is regulated, primarily by caspase-1. It plays a critical role in cellular adhesion regulating ICAM-1 expression in response to IL-1, TNF-α and other CYTOKINES. It also synergises with IL-12 in stimulating IFN-γ production. Soluble IL-18 RECEPTOR may be particularly interesting from an immunopharmacological point of view as it has lost its signalling domain and may therefore serve as a potent anti-inflammatory molecule.

Interleukin-17 family

The IL-17 family (IL-17A–F) includes CYTOKINES that share a similar protein structure, with four highly conserved cysteine residues critical to their three-dimensional shape, which is unique for this cytokine [28]. Production of IL-17A, which is characteristic for a specific subset of T helper $CD4^+$ LYMPHOCYTES called Th17 cells, places this cytokine as one of the most important regulators of autoimmune processes. IL-17 is particularly important as it regulates expression and function of numerous other pro-inflammatory signalling molecules. The role of IL-17 is also commonly associated with allergic responses. IL-17A, the best characterised member of this family, induces the production of many other CYTOKINES (such as IL-6, G-CSF, GM-CSF, IL-1β, TGF-β, TNF-α), CHEMOKINES (including IL-8, GRO-α and MCP-1) and PROSTAGLANDINS (PGE_2) discussed in this chapter. IL-17 RECEPTORS, binding particularly IL-17A, are expressed on fibroblasts, endothelial cells, epithelial cells, keratinocytes and MACROPHAGES [29]. As a result of these effects, the IL-17 family has been linked to many immune/autoimmune related diseases including RHEUMATOID ARTHRITIS, ASTHMA, SLE, ALLOGRAFT rejection and anti-tumour immunity.

Tumour necrosis factor-α and -β

These CYTOKINES are produced primarily in mononuclear PHAGOCYTES (TNF-α) and in LYMPHOCYTES (TNF-β) but also by numerous other cells. Activation of TOLL-LIKE RECEPTORS (TLR2 and TLR4) by LPS is the most commonly recognised intracellular pathway leading to production of TNF. TNF-α and -β bind with similar AFFINITY to the same cell surface RECEPTORS – TNFR 1 (p55) and TNFR 2 (p75). Therefore, their activities are very similar. The generic name of these CYTOKINES is based on tumour CYTOTOXIC effects, but their pharmacological use in the treatment of tumours is limited by severe side effects. TNF

is responsible for severe cachexia during chronic infections and cancer.

In endothelial cells these CYTOKINES induce expression of ADHESION MOLECULES (ICAM-1 and VCAM-1), and synthesis of PROSTACYCLIN and CYTOKINES. TNFs act as chemoattractants, as well as potent activators for NEUTROPHILS and MACROPHAGES. TNF-α causes fever and releases acute phase proteins. TNF and IL-1 produce many of the same pro-inflammatory responses, which include induction of COX and LIPOXYGENASE enzymes as well as the activation of B cells and T cells. It is finally important to point out that TNF is the primary mediator of haemodynamic changes during septic SHOCK through its negative inotropic effects as well as an increase in vascular permeability.

Transforming growth factor-α

TGF-α is a trophic regulator of cell proliferation and differentiation which is important in repair processes, it is involved in angiogenesis and in the organisation of extracellular matrix, it is chemotactic for MONOCYTES.

Platelet-derived growth factors

PDGFs cause proliferation of fibroblasts, vascular endothelial cells, smooth muscle. They are implicated in angiogenesis, atherosclerosis and possibly in chronic ASTHMA.

Interferons

IFNs constitute a group of inducible CYTOKINES that are synthesised in response to viral and other stimuli. There are three classes of IFN: termed type I (IFN-γ), type II (IFN-α and IFN-β) and type III (IFN-λ). IFN-α is not a single substance but a family of 15 proteins with similar activities. The IFNs have antiviral activity and IFN-γ has significant immunoregulatory function and only modest anti-viral activity. IFN-λ is also an antiviral cytokine but it signals through a distinct RECEPTOR complex, composed of the IFN-λR1 and IL-10R2 RECEPTOR chains. Thus, type III IFN, while being functionally an IFN, is structurally related to the IL-10 family. The antiviral effects of IFNs are achieved by inhibition of viral replication within infected cells as well as by stimulation of CYTOTOXIC LYMPHOCYTES and NK cells. All IFNs can be induced by other CYTOKINES, such as IL-1, IL-2, TNF, CSFs. IFN-α and IFN-β are produced in many cell types (MACROPHAGES, fibroblasts, endothelial cells, osteoblasts, etc.), and are strongly induced by viruses, and less strongly by other microorganisms and bacterial products. IFNs induce the expression of the major histocompatibility molecules (MHC classes I and II) that are involved in antigen presentation to T cells. IFNs also stimulate the expression of Fc RECEPTORS on GRANULOCYTES, promote the differentiation of myeloid cells and modulate the synthesis of CYTOKINES. IFN-γ is primarily made by T LYMPHOCYTES (T helper type 1), which may suggest that it is more of an INTERLEUKIN than an interferon. Indeed, it functions as an inhibitor of IL-4-dependent expression of low-AFFINITY IgE RECEPTORS, therefore inhibiting IgE synthesis.

Colony stimulating factors

These include IL-3 and granulocyte/macrophage (GM)-CSF and several other CYTOKINES. They regulate HAEMATOPOIESIS, are chemotactic for NEUTROPHILS, and activate NEUTROPHILS and MACROPHAGES.

Anti-inflammatory cytokines

It is important to point out that apart from pro-inflammatory actions, some CYTOKINES may inhibit inflammatory processes. These include IL-1ra, mentioned above, as well as TGF-β or the IL-10 family, which includes IL-10, -19, -20, -22 and -24.

Intracellular signalling by cytokine receptors

Binding of CYTOKINES to their RECEPTORS leads to the activation of cytoplasmic tyrosine kinases. Janus kinases (JAKs), a recently described family of four related cytoplasmic protein tyrosine kinases, further transfer cytokine signalling. There are four JAKs: JAK1, JAK2, JAK3, and TYK2, which transduct signals from cytokine RECEPTORS to effector mechanisms. On binding of the cytokine, JAKs bind to the RECEPTOR and mediate tyrosine kinase activity and phosphoryla-

tion of the RECEPTOR and of RECEPTOR-associated JAKs (Fig. 5). The next step in signal transduction involves tyrosine phosphorylation of signal transducers and activators of transcription (STATs) in the cytoplasm. Upon activation, STATs become phosphorylated, form homodimers, and migrate to the nucleus, where they bind to regulatory sequences in the promoters of cytokine-responsive genes, e.g. ICAM-1 or other cytokine genes. In summary, cytokine signalling is based on a relatively small number of redundant tyrosine kinases. For instance, JAK-1 and JAK-3 transduct signals from CYTOKINES such as IL-2 or IL-4, while JAK-2 is involved in IL-3, IL-6 and GM-CSF signalling. Similarly, the number of STATs is low when compared to the number of CYTOKINES. Therefore, one can conclude that some additional mechanisms will guide different responses to various CYTOKINES. An additional pathway used by many cytokine RECEPTORS includes RAS-dependent cascades. In this signal transduction cascade Ras, Raf-1, MAP/Erk kinase kinase (MEKK) and finally MITOGEN-activated protein kinases (MAPK) are sequentially activated and lead to regulation of cellular proliferation by GROWTH FACTORS and responses to IL-2 or IL-3. The activation of other signalling pathways, like insulin RECEPTOR substrates (IRS-1, IRS-2), can also mediate some other biological activities of CYTOKINES, including proliferation and regulation of APOPTOSIS. In conclusion, it becomes apparent that different combinations of the signalling mechanisms described above will lead to many distinct responses to different CYTOKINES.

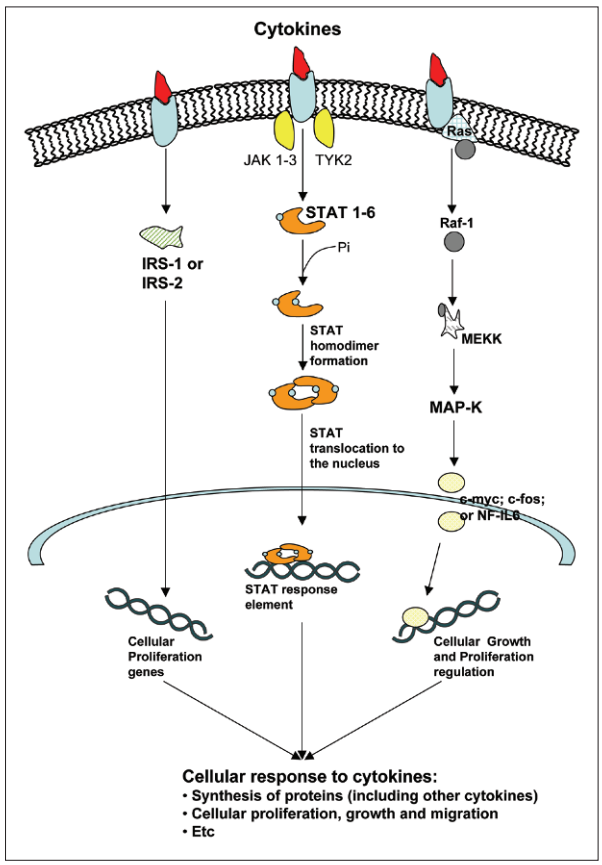

FIGURE 5
Cytokine-induced intracellular signalling

Chemokines and their intracellular signalling

Chemokines are a family of 8–12-kDa molecules, which induce CHEMOTAXIS of MONOCYTES, LYMPHOCYTES, NEUTROPHILS, other GRANULOCYTES as well as vascular smooth muscle cells and variety of other cells (see also chapter A5). There are 47 CHEMOKINES, sharing 30–60% homology. Chemokines are characterised by the presence of three to four conserved cysteine residues. The new classification of CHEMOKINES is based on the positioning of the N-terminal cysteine residues (Tab. 4). Chemokines are usually secreted proteins, except for fractalkine (CX3CL1), which is the only membrane-bound chemokine, acting as an ADHESION MOLECULE. Most CHEMOKINES play roles in recruiting and activating immune cells to the site of INFLAMMATION, while others are important in maintaining homeostasis within the IMMUNE SYSTEM (housekeeping CHEMOKINES: CCL5, CCL17-19, 21, 22, 25, 27, 28, CXCL13, CXCL14). Homeostatic CHEMOKINES are expressed in an organ-specific manner, while inflammatory CHEMOKINES can be produced by multiple cell types.

Their effects are achieved through interaction with chemokine RECEPTORS. There are 18 chemokine RECEPTORS currently known, therefore some RECEPTORS may bind several ligands, which leads to overlapping functions of known CHEMOKINES. Moreover, a single

cell may express several chemokine RECEPTORS. One of the key features of chemokine RECEPTORS, owing to their heptahelical transmembrane structure, is their ability to signal through different intracellular signalling pathways. Binding of chemokine to the RECEPTOR leads to activation of Gα protein and binding of GTP. The Gα subunit activates Src kinases and subsequently MAPKs and protein kinase B (PKB). During activation of Gα protein, a Gβγ complex is liberated and may independently lead to activation of PKB and MAPKs (*via* PI_3), PKC activation *via* phospholipase C (PLC) and finally through Pyk-2 [30]. These pathways lead to up-regulation of membrane INTEGRINS and initiate ROLLING and adhesion of cells as well as their conformational changes. Some of these intracellular pathways (in particular PLC activation) may then lead to an increase in intracellular calcium and its consequences, including DEGRANULATION, NOS activation, etc., within the TARGET cells.

It is difficult to accurately describe the relative importance of individual CHEMOKINES. The largest number of studies was conducted on the actions of IL-8 as the most important chemoattractant for POLYMORPHONUCLEAR LEUKOCYTES, although it appears late during the inflammatory response. Other well investigated members of this family include CCL3 (MIP-1α) or RANTES (CCL5).

Apart from effects on CHEMOTAXIS, CHEMOKINES have direct and indirect effects on T cell differentiation into Th1 or Th2 subclasses, therefore regulating the nature of immune responses [27, 30].

Due to the critical role of CHEMOKINES in INFLAMMATION, interest has focused on potential therapeutic effects of inhibiting their activity. Both peptide antagonists as well as gene transfer approaches have been successfully used to inhibit INFLAMMATION in various animal models (e.g. allergic INFLAMMATION models or ApoE-knockout atherosclerosis prone mice).

Neuropeptides

NEUROPEPTIDES are released from sensory neurons and in some tissues they contribute to inflammatory reactions. For example, substance P and other tachykinins produce smooth muscle contraction, mucus secretion, cause vasodilation and increase vascular permeability. "Calcitonin gene-related peptide" (CGRP) is a potent vasodilator, acting on CGRP RECEPTORS leading to activation of adenylate cyclase. The overall pattern of effects of tachykinins is similar, although not identical, to the pattern seen with kinins.

Tachykinins

The mammalian tachykinins comprise three related peptides: substance P (SP), neurokinin A (NKA) also called substance K, and neurokinin B (NKB). They occur mainly in the nervous system, particularly in nociceptive sensory neurons and in enteric neurons. They are released as neurotransmitters, often in combination with other mediators. SP and NKA are encod-

TABLE 4. CLASSES OF CHEMOKINES

Subfamily	Chemokines	Characteristics
C-X-C	CXCL 1–16, includes IL-8 (CXCL8)	First two cysteines separated by a variable amino acid
C-C	CCL1-28 (include MIP-1 MCP and RANTES)	First two cysteines are adjacent to each other
C	XCL 1 (lymphotactin) and XCL 2	Lacks first and third cysteine residue
CX3CL1	CX3CL1 (Fractalkine)	Two N-terminal cysteine residues separated by 3 variable amino acids

ed by the same gene and they have a similar distribution. Three distinct types of tachykinin RECEPTOR are known: NK_1, NK_2, and NK_3. They are selective for three endogenous tachykinins with the following AFFINITY: for NK_1, SP>NKA>NKB; for NK_2, NKA>NKB>SP; and for NK_3, NKB>NKA>SP RECEPTOR. RECEPTOR cloning has shown that tachykinin RECEPTORS belong to a family of G protein-coupled RECEPTORS. Several potent antagonists of NK_1 and NK_2 and NK_3 RECEPTORS have been discovered [31], and novel therapeutic agents for various disease states (e.g. pain, ASTHMA, arthritis, headache) may be developed.

CGRP differs from other tachykinins. It is coded for by the calcitonin gene, which also codes for calcitonin itself. Differential splicing allows cells to produce either procalcitonin (expressed in thyroid cells) or pro-CGRP (expressed in neurons) from the same gene. CGRP is found in non-myelinated sensory neurons and it is a potent inducer of neurogenic INFLAMMATION.

Kinins

Kinins are polypeptides with vasodilator/hypotensive, thrombolytic, pro-inflammatory and algesic (painful) actions. The two best known kinins are BRADYKININ and kallidin and they are referred to as plasma kinins. Since 1980, when Regoli and Barabe divided the kinin RECEPTORS into B_1 and B_2 classes, first- and second-generation kinin RECEPTOR antagonists have been developed, leading to a much better understanding of the actions of kinins.

BRADYKININ is a nonapeptide; kallidin is a decapeptide and has an additional lysine residue at the N-terminal position. These two peptides are formed from a class of α-2 globulins known as kininogens (Fig. 6). There are two kininogens: high molecular weight (HMW) and low molecular weight (LMW) kininogen which are products of a single gene that arises by alternative processing of mRNA. The highly specific proteases that release BRADYKININ and kallidin from the kininogens are termed kallikreins. Two distinct kallikreins, formed by different activation mechanisms from inactive prekallikreins, act on the kininogens. One of these is plasma kallikrein and the other is tissue kallikrein. LMW kininogen is a SUBSTRATE only for the tissue kallikrein and the product is kallidin, while HMW kininogen is cleaved by plasma and tissue kallikrein to yield BRADYKININ and kallidin, respectively.

Kallidin is similar in activity to BRADYKININ and need not be converted to the latter to exert its effects. However, some conversion of kallidin to BRADYKININ occurs in plasma due to the activity of plasma aminopeptidases.

The half-life of kinins in plasma is about 15 seconds and concentrations of kinins found in the circulation are within the picomolar range. BRADYKININ is inactivated by a group of enzymes known as kininases. The major catabolising enzyme in the lung and in other vascular beds is kininase II, which is identical to peptidyl dipeptidase, known as angiotensin converting enzyme (ACE). Kininase II is inhibited by captopril, resulting in an increased concentration of circulating BRADYKININ, which contributes substantially to the antihypertensive effect of captopril. On the other hand, kininase I is arginine carboxypeptidase and has a slower action than kininase II. It removes the C-terminal arginine residue producing des-Arg^9-BRADYKININ or des-Arg^{10}-kallidin, which are themselves potent B_1-kinin RECEPTOR agonists.

There are at least two distinct RECEPTORS for kinins, B_1 and B_2. The classical, constitutive BRADYKININ RECEPTOR, now designated the B_2 RECEPTOR, selectively binds BRADYKININ and kallidin and mediates a majority of the effects of BRADYKININ and kallidin in the absence of INFLAMMATION, such as the release of PGI_2 and NO from endothelial cells. On the other hand, inducible B_1 RECEPTORS are up-regulated by INFLAMMATION. They bind des-Arg metabolites of BRADYKININ and kallidin. In contrast to B_1 RECEPTORS, the signalling mechanism of B_2 RECEPTORS has been well characterised. The B_2 RECEPTOR is coupled to G protein and activates both PLA_2 and PLC. While stimulation of the former liberates AA from phospholipids, with its subsequent oxidation to a variety of pro-inflammatory EICOSANOIDS, the activation of PLC through IP_3 and DAG leads directly to pro-inflammatory effects.

During the last decade the existence of other types of kinin RECEPTORS (B_3, B_4, B_5) has been suggested. However, recent studies indicate that some

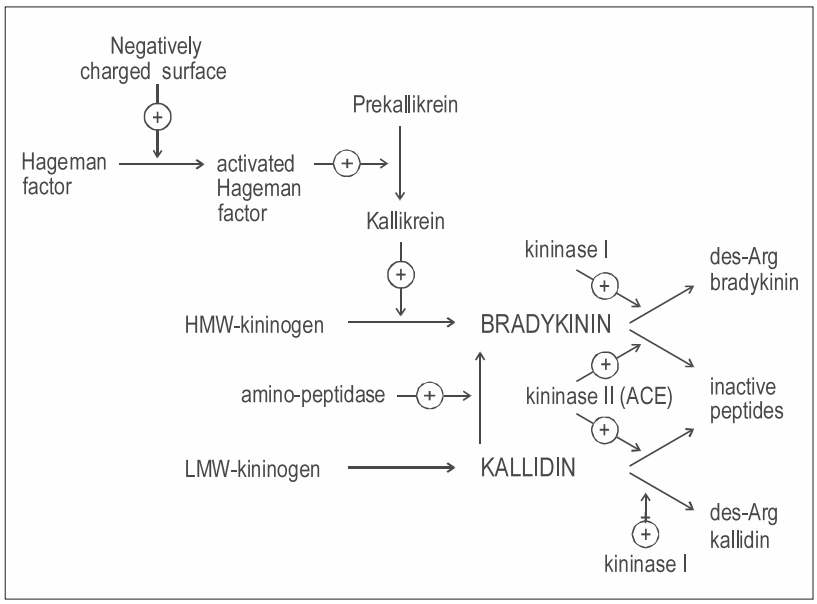

FIGURE 6
The formation and metabolism of kinins

of them may actually represent functions of the B_2 RECEPTOR [19].

Kinins are among the most potent vasodilators known, acting on arteriolar beds of the heart, liver, skeletal muscle, kidney, intestines, and ovaries. They are claimed to play a minor role in the regulation of blood pressure in health individuals, but they play a major vasodepressor regulatory role most likely mediated by arterial endothelium in hypertensive patients [32]. Indeed, kinins contract veins and non-vascular smooth muscle, such as gastrointestinal and bronchial muscle. BRADYKININ and kallidin have similar contracting properties. At the level of the capillary circulation, kinins increase permeability and produce oedema. Stimulation of B_1 RECEPTORS on inflammatory cells such as MACROPHAGES can elicit the production of the inflammatory mediators such as IL-1 and TNF-α [33]. Kinins are also potent pain-inducing agents in both the viscera and skin. In acute pain, B_2 RECEPTORS mediate BRADYKININ-induced algesia. The pain of chronic INFLAMMATION appears to involve an increased expression of B_1 RECEPTORS.

As in the case of other autacoids, the therapeutic interest in kinins has focused particularly on attempts to modulate their formation or metabolism *in vivo* [34]. Blockade of kinin formation with a kallikrein inhibitor, aprotynin (Trasylol), has been used with some success to treat acute pancreatitis, carcinoid syndrome or Crohn's disease. Experimentally, progress has been made in the development of selective antagonists of kinins. Currently, they are not available for clinical use. However, recent studies indicate that kinin RECEPTOR antagonists might be useful for the treatment of patients with septic SHOCK, pancreatitis-induced hypotension bronchial ASTHMA, rhinovirus-induced symptoms and in treating pain.

Nitric oxide

In animal tissues, NITRIC OXIDE (NO) is generated enzymatically by NO synthases (NOS). The three NOS isoenzymes (neuronal, endothelial and inducible) are flavoproteins, which contain tetrahydrobiopterin and haem, and they are homologous with cytochrome P450 reductase [35]. Isoenzymes of NOS act as dioxygenases using molecular oxygen and NADPH to transform L-arginine to L-citrulline and NO (Fig. 7). NO formed by endothelial constitutive NOS (eNOS) is responsible for maintaining low vascular

tone and preventing LEUKOCYTES and platelets from adhering to the vascular wall. eNOS is also found in renal mesangial cells. NO formed by neuronal constitutive NOS (nNOS) acts as a neuromodulator or neuromediator in some central neurons and in peripheral "non-adrenergic non-cholinergic" (NANC) nerve endings. NO formed by inducible NOS (iNOS) in MACROPHAGES and other cells plays a role in the inflammatory response.

NO was discovered by Furchgott and Zawadzki as "endothelium-derived relaxing factor" (EDRF) [36]. It soon became obvious that EDRF, like nitroglycerine, activates soluble guanylate cyclase in vascular smooth muscle by binding to its active haem centre. The rise in cyclic GMP achieved is responsible for vasodilatation and for other physiological regulatory functions of NO.

The activities of constitutive nNOS and eNOS are controlled by intracellular calcium/calmodulin levels. For instance, nNOS in central neurons is activated by glutamate binding to NMDA RECEPTORS with a subsequent rise in $[Ca^{2+}]_i$ due to opening of voltage calcium channels, whereas eNOS is activated by blood shear stress or stimulation of endothelial muscarinic, purinergic, kinin, substance P or thrombin RECEPTORS. This triggers an increase in $[Ca^{2+}]_i$ at the expense of the release of Ca^{2+} from endoplasmic reticulum.

Calcium ionophores (e.g. A23187) and polycations (e.g. poly-L-lysine) cause a rise in $[Ca^{2+}]_i$ and activate eNOS thereby bypassing the RECEPTOR mechanisms.

In contrast to the constitutive isoforms of NOS, iNOS does not require a rise in $[Ca^{2+}]_i$ to initiate its activity. In MACROPHAGES, MONOCYTES and other cells the induction of iNOS and the presence of L-arginine are sufficient to initiate the generation of NO. Induction of iNOS can be initiated by IFN-γ, TNF-α or IL-1. However, the best recognised inducer is LPS or ENDOTOXIN from *Escherichia coli*, which is known to be responsible for the development of Systemic Inflammatory Response Syndrome (SIRS) in the course of sepsis due to gram-negative bacteria. Myeloid cells express a RECEPTOR for LPS on their cell membrane, m-CD14 protein. LPS, using an "LPS binding protein" (LBP), is anchored to m-CD14 and then triggers a chain of protein phosphorylation, which eventually leads to the activation of the major transcription protein NF-κB. This is responsible for transcription of the message to make iNOS. In cells that lack m-CD14, the induction of iNOS is achieved by a complex of soluble s-CD14 with LBP and LPS itself. In a similar manner, LPS can also induce COX-2. Although NO fulfils more paracrine than autoendocrine functions, in the case of iNOS, large amounts of locally formed NO may inhibit iNOS itself as well as COX-2, in a negative feedback reaction. Glucocorticosteroids and some CYTOKINES, such as TGF-β, IL-4 or IL-10, inhibit the induction of iNOS.

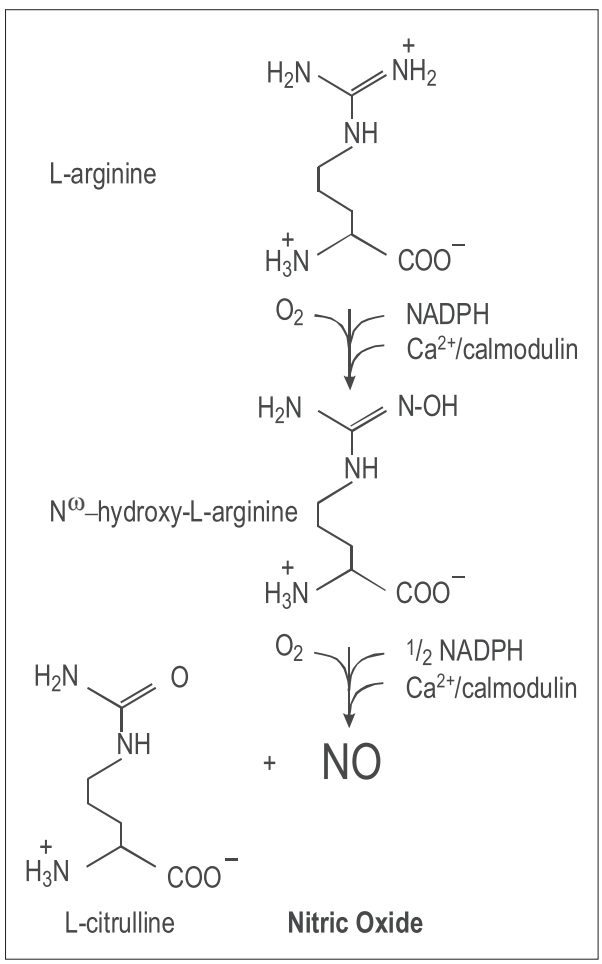

FIGURE 6
The synthesis and metabolism of nitric oxide (NO).

Nitric oxide as an effector of inflammation

Kinetics of NO production by iNOS differ greatly from production by eNOS or nNOS (Fig. 8) [37]. Inducible NOS produces very large, toxic amounts of NO in a sustained manner, whereas consititutive NOS isoforms produce NO within seconds and its activities are direct and short acting. There are multiple intracellular mechanisms through which NO may act as an inflammatory mediator [38]. Low levels of NO produced by constitutive synthases primarily interact directly with positively charged metal ions of guanylate cyclase, cytochrome P450 and NOS itself. Activation of guanylate cyclase leads to an increase in intracellular cyclic guanosine monophosphate (cGMP), which in turn activates cGMP-dependent protein kinases, which mediate NO actions including vasorelaxation, increase of vascular permeability, and anti-proliferative, anti-platelet and anti oxidant effects of NITRIC OXIDE. Recent data have also indicated that NO produced by constitutive NOS enzymes may be involved in immune regulation of T helper cell proliferation and cytokine production.

During the course of an inflammatory response, the large amounts of NO formed by iNOS surpass the physiological amounts of NO which are usually made by nNOS or eNOS. The functions of iNOS-derived NO are also different. In immunologically or chemically activated MACROPHAGES, NO kills microorganisms and destroy macromolecules. NO formed by constitutive isoforms of NOS, is stored as a nitrosothiol in albumin and acts physiologically as *N*-nitrosoglutathione and *N*-nitrosocysteine. Eventually, within a few seconds, NO is oxidised to nitrites or nitrates. Large amounts of "inflammatory NO" from myeloid cells are usually generated side by side with large amounts of SUPEROXIDE ANION (O_2^-). These two can form PEROXYNITRITE ($ONOO^-$), which mediates the CYTOTOXIC effects of NO, such as DNA damage, LDL oxidation, ISOPROSTANE formation, tyrosine nitration, inhibition of aconitase and mitochondrial respiration. The discovery of this reaction opens new possibilities for the therapeutic use of superoxide dismutase (SOD). Indeed SOD mimetics have been successfully used to limit the extent of INFLAMMATION. Interestingly, overstimulation of NMDA RECEPTORS by glutamate may activate nNOS to such an extent that NO itself exerts neurotoxic properties. NO formed by eNOS seems to be mostly cytoprotective, possibly due to its unusual redox properties.

Large amounts of NO and $ONOO^-$ may TARGET numerous proteins and enzymes critical for cell survival and signalling. These include signalling molecules involved in cytokine signalling like JAK or STAT proteins, NK-κB/IκB pathway as well as MAPK, some G proteins and transcription factors. Nitration of cysteines in these proteins may lead to their activation or inactivation.

NO is scavenged by haemoglobin, methylene blue and pyocyanin from *Pseudomonas coereleus*. These last two are also claimed to be inhibitors of guanylate cyclase. GLUCOCORTICOIDS selectively inhibit the expression of iNOS. Arginine analogues, such as L-N^G-monomethyl arginine (L-NMMA) and L-N^G-nitro-arginine methyl ester (L-NAME) inhibit inducible and constitutive NOS isoforms non-selectively. Selective iNOS inhibitors (e.g. alkylisothioureas or aminoguanidines) are being intensively investigated in the hope that selective inhibition of iNOS may prevent development of SIRS (systemic inflammatory response syndrome) or MODS (multiple organ dysfunction syndrome). Indeed, overproduction of NO by iNOS during septicaemia is claimed to be responsible for irreversible arterial hypotension, vasoplegia (loss of responses to noradrenaline), lactic acidosis, suffocation of tissues, their necrosis and APOPTOSIS. However, it is important to remember that NO made by iNOS is of benefit to the host defence reaction by contributing to microbial killing.

Moreover, NO generated by eNOS is essential to maintain tissue perfusion with blood, to offer cytoprotection in the pulmonary and coronary circulation against toxic lipids, which are released by LPS, and to preserve red cell deformability, which becomes reduced in septicaemia [39]. Preliminary clinical experience with L-NMMA has been reasonably encouraging, as long as a low dose of the NOS inhibitor is used. In animal models of endotoxic SHOCK, non-selective NOS inhibitors were reported to decrease cardiac output, to increase pulmonary pressure, to decrease nutritional flow to organs, to damage gastric mucosa and to increase mortality rate. On the other hand, inhalation of NO gas (10 ppm) in septic patients has been found to pre-

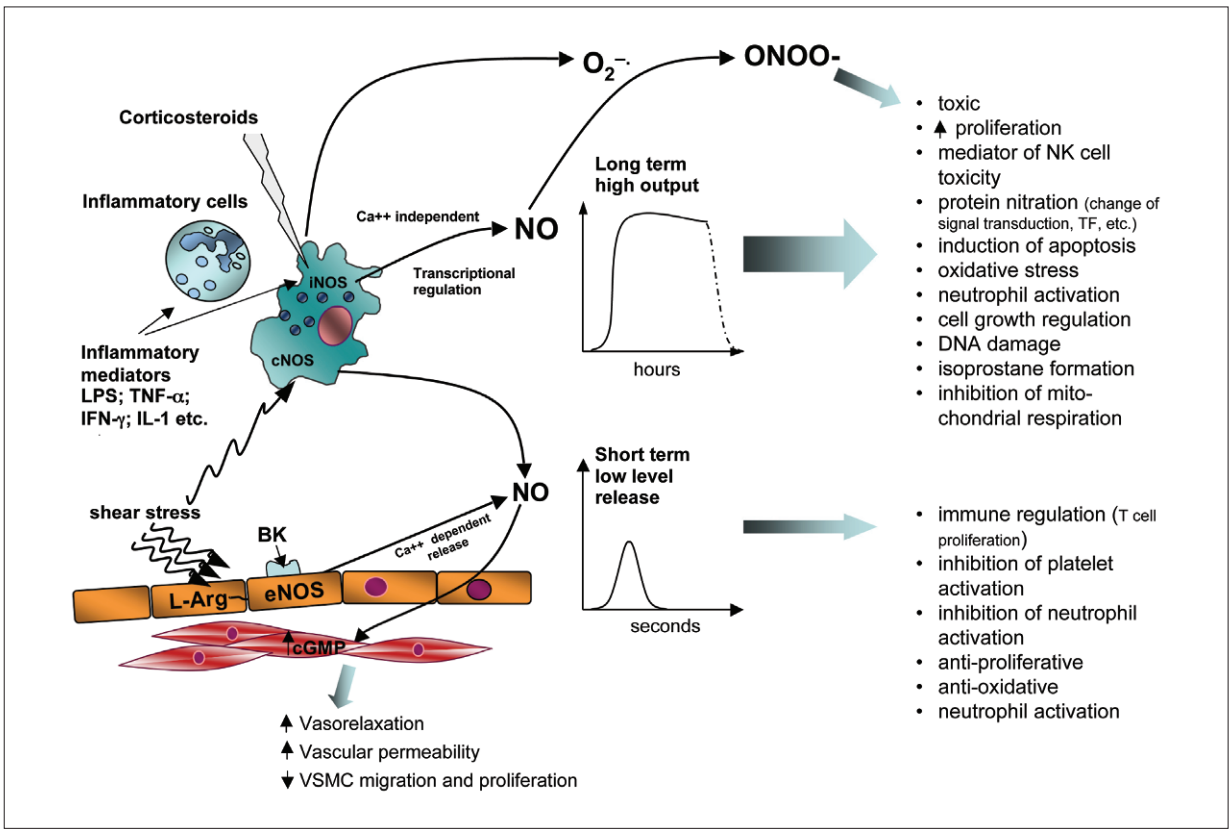

FIGURE 8
Differences between kinetics of nitric oxide generation by eNOS and iNOS

vent the mismatch of the ventilation/perfusion ratio in their lung. The exact role of NO in various stages of sepsis, SIRS and MODS still awaits further elucidation and evaluation.

Nitric oxide in immune regulation

The exact role of NO in immune regulation is also unclear. Initial mouse studies suggested that APC-derived NO may inhibit T cell proliferation, particularly of the Th1 subset of T helper cells. Mouse Th1 cells were also shown to produce NO, suggesting that the above mechanism is a part of a negative feedback process. In this way, NO would inhibit Th1 and therefore promote Th2 type cytokine responses, leading to humoral and allergic responses. Subsequent studies, however, indicate that both Th1 and Th2 produce similar amounts of NO, and both subsets respond similarly to NO. NO-induced changes in lymphocyte proliferation seem to be dependent more on the effects on the cell cycle proteins than due to changes in cytokine profile [37].

It is also important to recognise that cells that produce NO protect themselves against its toxic actions [40]. Recent studies show that GSH-GSSG anti-oxidative systems protect MACROPHAGES against large amounts of NO generated by iNOS. In addition, endothelial cells appear not to be primary responders to NO produced by eNOS because increases in intracellular calcium which mediate eNOS activation are also able to inhibit guanylate cyclase activity.

Reactive oxygen species

ROS production plays an important role in modulation of inflammatory reactions. Major ROS produced within the cell are SUPEROXIDE ANION, hydrogen peroxide and hydroxyl radical [38]. Extracellular release of large amounts of SUPEROXIDE ANION produced by the RESPIRATORY BURST in LEUKOCYTES is an important mechanism of pathogen killing and also leads to endothelial damage, resulting in an increased vascular permeability as well as cellular death (see also chapter A6). However, vast evidence has implicated intracellular ROS production as a key player in modulation of the release of other mediators of INFLAMMATION. This is related mainly to the constitutive expression of NAD(P)H oxidases (termed NOXs – non-phagocytic oxidases) in various tissues [37]. ROS produced by this family of enzymes can regulate ADHESION MOLECULE expression on endothelium and inflammatory cells, thus regulating cellular recruitment to the sites of INFLAMMATION. They also increase chemokine and cytokine expression. At least some of these effects result from the ability of ROS (in particular H_2O_2) to stimulate MAPK activities, which lead to activation of several transcription factors. It is possible that intracellular ROS may act as second messengers in inflammatory signal transduction [37].

Inflammatory CYTOKINES (like TNF-α) may in turn increase NAD(P)H oxidase activity and expression which closes the vicious circle of INFLAMMATION. While loss of NAD(P)H oxidase activity in cells leads to diminished INFLAMMATION in the vascular wall, several humoral factors may affect constitutive NAD(P)H oxidase expression in the vascular wall and therefore intracellular ROS production. These include angiotensin II, endothelins, high glucose or high cholesterol levels. Their effects on baseline ROS production may therefore mediate modulatory effects of these factors on INFLAMMATION which traditionally were not associated with INFLAMMATION. Interestingly, T and B LYMPHOCYTES at various stages of their development and activation express NADPH oxidases, mainly classical gp91phox containing NADPH OXIDASE – Nox2 (mature T cells) and a calcium dependent – Nox5 (during development).

Accordingly, attempts have been undertaken to inhibit intracellular ROS production in order to limit inflammatory responses. Apocynin, an NAD(P)H oxidase activation inhibitor has been successfully used in limiting INFLAMMATION in animal models of RHEUMATOID ARTHRITIS, while decoy peptides preventing association of NAD(P)H oxidase subunits were shown to be effective in INFLAMMATION related to atherosclerosis.

Amines

Histamine

HISTAMINE, 2-(4-imidazolyl)-ethyl-amine, is an essential biological amine in INFLAMMATION and ALLERGY. It is found mostly in the lung, skin and in the gastrointestinal tract. It is stored together with macroheparin in granules of mastocytes or BASOPHILS (0.01–0.2 pmoles per cell), from which it is released when COMPLEMENT COMPONENTS C3a and C5a interact with specific RECEPTORS, or when antigen interacts with cell-fixed IgE. These trigger a secretory process that is initiated by a rise in cytoplasmic Ca^{2+} from intracellular stores. Morphine and tubocurarine release HISTAMINE by a non-RECEPTOR action. Agents that increase cAMP formation inhibit HISTAMINE secretion, so it is postulated that, in these cells, c-AMP-dependent protein kinase is an intracellular restraining mechanism. Replenishment of the HISTAMINE content of MAST CELLS or BASOPHILS after secretion is a slow process, whereas turnover of HISTAMINE in the gastric histaminocyte is very rapid.

HISTAMINE is synthesised from histidine by a specific decarboxylase and metabolised by histaminases and/or by imidazole *N*-methyltransferase. HISTAMINE exerts its effects by acting on H_1-, H_2- or H_3-RECEPTORS on TARGET cells [41]. It stimulates gastric secretion (H_2), contracts most of the smooth muscle other than that of blood vessels (H_1), causes vasodilatation (H_1), and increases vascular permeability by acting on the post-capillary venules [42]. Injected intradermally, HISTAMINE causes the triple response: local vasodilatation and wheal by a direct action on blood vessels and the surrounding flare, which is

due to vasodilatation resulting from an axon reflex in sensory nerves thereby releasing a peptide mediator [42]. Of many functions of HISTAMINE, the stimulation of gastric acid secretion, and mediation of type 1 HYPERSENSITIVITY, such as urinary and hay fever, are among the most important. The full physiological significance of the H_3-RECEPTOR has yet to be established [43]. HISTAMINE may also be involved in T helper cell immune regulation (extensively reviewed in [44]).

5-Hydroxytryptamine

5-Hydroxytryptamine (5-HT, SEROTONIN) was originally isolated and characterised as a vasoconstrictor released from platelets in clotting blood. 5-HT occurs in chromaffin cells and enteric neurons of the gastrointestinal tract, in platelets and in the CENTRAL NERVOUS SYSTEM. It is often stored together with various peptide hormones, such as SOMATOSTATIN, substance P or "vasoactive intestinal polypeptide" (VIP). The biosynthesis and metabolism of 5-HT closely parallels that of CATECHOLAMINES, except the precursor for decarboxylase of aromatic amino acids is 5-hydroxytryptophan instead of tyrosine (Fig. 9). 5-HT is inactivated mainly by the monoamine oxidases A or B (MAO A or B) to 5-hydroxyindoleacetic acid (5-HIAA), which is excreted in the urine. Some 5-HT is methylated to 5-methoxytryptamine, which is claimed to be involved in the pathogenesis of affective disorders.

The actions of 5-HT are numerous and complex, showing considerable variation between species [45]. For instance, in the inflammatory response, 5-HT seems to be more important in rats than in humans. 5-HT is known to increase gastrointestinal motility and to contract bronchi, uterus and arteries, although 5-HT may also act as a vasodilator through endothelial release of NO. In some species, 5-HT stimulates platelet aggregation, increases microvascular permeability and stimulates peripheral nociceptive nerve endings. A plethora of pathophysiological functions proposed for 5-HT includes control of peristalsis, vomiting, haemostasis, INFLAMMATION and sensitisation of nociceptors by peripheral mechanisms or control of appetite, sleep, mood, stereotyped behaviour and pain perception by central mechanisms. Clinically, disturbances in the 5-HT regulation system have been proposed in migraine, carcinoid syndrome, mood disorders and anxiety [45].

These diverse actions of 5-HT are not mediated through one type of RECEPTOR. The amino acid sequence for many 5-HT RECEPTOR subtypes has been determined by cloning, and the transduction mechanisms to which these RECEPTORS are coupled have been explained. The basic four types of RECEPTORS are 5-$HT_{1–4}$. 5-HT_1 and 5-HT_2 RECEPTORS are further subdivided into A, B and C subtypes [46]. Types 1, 2 and 4 are G protein-coupled RECEPTORS, type 3 is a LIGAND-gated cation channel. 5-HT_1 RECEPTORS occur mainly in the CNS (all subtypes) and in blood vessels (5-HT_{1D} subtype). 5-HT_{1B} and 5-HT_{1D} RECEPTORS

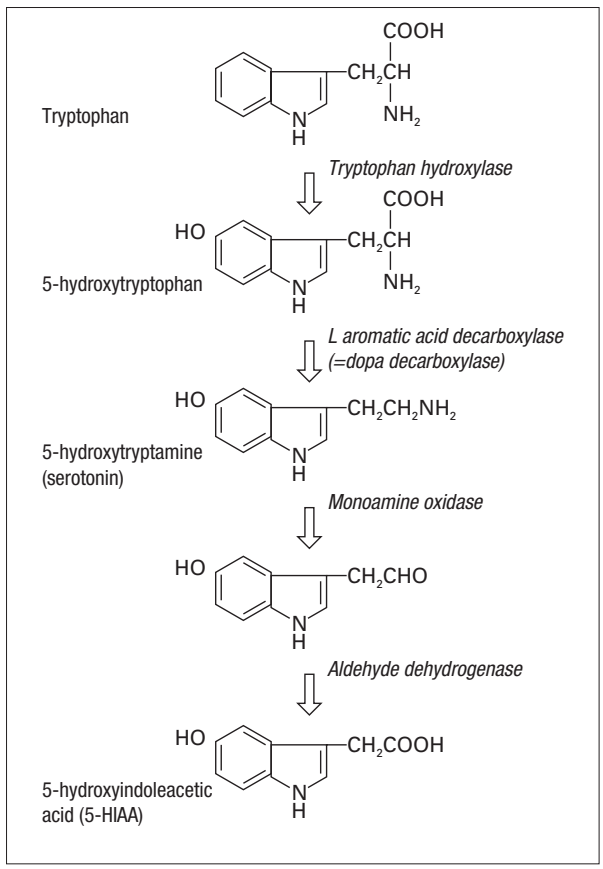

FIGURE 9
The synthesis and breakdown of 5-HT

appear to be involved, at least in part, in the modulation of neurogenically induced (following electrical, chemical or mechanical depolarisation of sensory nerves) vascular INFLAMMATION. 5-HT_2 RECEPTORS (5-HT_{2A} subtype being functionally the most important) are distributed more in the periphery than in the CNS and they are linked to phospholipase C, which catalyses phosphatidylinosytol hydrolysis. The role of 5-HT_2 RECEPTORS in normal physiological processes is probably a minor one, but it becomes more prominent in pathological conditions, such as ASTHMA, INFLAMMATION or vascular thrombosis. 5-HT_3 RECEPTORS occur particularly on nociceptive sensory neurons and on autonomic and enteric neurons, on which 5-HT exerts an excitatory effect and evokes pain when injected locally.

Catecholamines

It has become increasingly recognised that the release of CATECHOLAMINES at autonomic nerve endings and from the adrenal medulla may modulate the function of immunocompetent cells (see also chapter A11). The major LYMPHOID ORGANS (spleen, lymph nodes, thymus, and intestinal PEYER'S PATCHES) are extensively supplied by noradrenergic sympathetic nerve fibres. Sympathetic nervous system innervation of these LYMPHOID ORGANS as well as the presence of adrenergic and dopamine RECEPTORS on immune cells, provide the channels for noradrenergic signalling to LYMPHOCYTES and MACROPHAGES by sympathetic nerves [47]. CATECHOLAMINES have a wide range of direct effects on immune cells, particularly on MACROPHAGES and LYMPHOCYTES. Stimulation of β-adrenergic RECEPTORS on LPS-pretreated MACROPHAGES prevents the expression and release of pro-inflammatory TNF-α and IL-1, while the release of anti-inflammatory IL-10 is augmented. On the other hand, α-adrenergic stimulation augments phagocytic and tumoricidal activity of MACROPHAGES. CATECHOLAMINES acting through β-adrenergic and dopaminergic RECEPTORS, which are linked to adenylate cyclase through cyclic-AMP, modulate the function of immune cells. An increase in intracellular cyclic-AMP inhibits lymphocyte proliferation and production of pro-INFLAMMATORY CYTOKINES. The demonstration of the presence of α_2-, β-adrenergic, D1 and D2 RECEPTORS on various immune cells has recently provided the basis for regulation of cytokine production, specifically INTERLEUKINS and TNF, by these RECEPTORS in response to LPS [42]. Vasopressor and inotropic CATECHOLAMINES seem to have potent immunomodulating properties which, as yet, have not been adequately explored and may contribute to the therapeutic effects of dobutamine or dopexamine in the treatment of septic SHOCK and SIRS.

Summary

INFLAMMATION is a protective response of the macro-organism to injury caused by trauma, noxious chemicals or microbiological toxins. This response is intended to inactivate or destroy invading organisms, remove irritants, and set the stage for tissue repair. The inflammatory response consists of immunological and non-immunological reactions. The latter are triggered by the release from injured tissues and migrating cells of lipid-derived autacoids (e.g. EICOSANOIDS or PAF), large peptides (e.g. IL-1 and CYTOKINES), small peptides (e.g. BRADYKININ), and amines (HISTAMINE or 5-HT). These constitute the chemical network of the inflammatory response and result in clinical and pathological manifestations of INFLAMMATION.

Prostanoids, as autacoids, are involved in virtually every stage of INFLAMMATION. They regulate vascular tone and permeability (PGs), induce platelet aggregation and thrombus formation (TX) and are involved in the pathogenesis of pain and fever (PGs) accompanying INFLAMMATION. The recently discovered LIPOXINS are important regulators of inflammatory reactions. PAF, cytokine and chemokine groups as well as kinins also play crucial pro-inflammatory roles. Recent studies have shed more light on our understanding of intracellular signalling mechanisms involved in the responses to pro-INFLAMMATORY CYTOKINES such as IL-1, TNF, TGF and INTERFERONS. TOLL-LIKE RECEPTORS contribute to the mediation of effects of components of micro-organisms on innate and ADAPTIVE IMMUNITY.

NO and ROS not only act as important effectors, causing damage to invading micro-organisms (NO from iNOS or SUPEROXIDE ANION) but may also be very important in immunoregulation, in part by regulating redox-sensitive genes. Co-ordinated pharmacological interventions, which would modify different parallel pathways in the inflammatory cascade, are needed to treat inflammatory diseases.

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Immune response in human pathology: Infections caused by bacteria, viruses, fungi, and parasites

Jan Verhoef, Kok van Kessel and Harm Snippe

Infections

In the middle of the 19th century, it became clear that micro-organisms could cause disease. Effective treatment, however, was not possible at that time; prevention and spread of infectious diseases depended solely on proper hygienic means. At the beginning of the 20th century, passive and active vaccination procedures were developed against a number of these PATHOGENIC MICRO-ORGANISMS to prevent the diseases in question (rabies, diphtheria, tetanus, etc.). Thanks to the discovery of antimicrobial chemicals (by Paul Ehrlich) and antibiotics (by Sir Alexander Fleming), the threat of infectious diseases seemed to be minimised. Large scale vaccination programmes against childhood diseases (diphtheria, whooping cough and polio), started in the early 1950s, raised hopes of finally being able to eradicate these diseases from the planet. This approach was successful for smallpox (1980). However, new infectious diseases have emerged [e.g., *Legionella*, HUMAN IMMUNODEFICIENCY VIRUS (HIV), *Helicobacter*, SARS, etc.] and new vaccines and antibiotics are needed. Furthermore, due to intensive medical treatment with antibiotics and immunosuppressive drugs, hospital infections are a growing problem. Bacteria hitherto deemed harmless are causing OPPORTUNISTIC INFECTIONS in immunocompromised patients. The pathogens have developed resistance to many antibiotics and sometimes no effective antibiotics are available to treat these patients.

To make the story even more serious, man is surrounded and populated by a large number of different non-PATHOGENIC MICRO-ORGANISMS. In the normal, healthy situation, there is a balance between the offensive capabilities of micro-organisms and the defences of the human body. The body's defences are based on vital non-specific and specific immunological defence mechanisms. An infection means that the micro-organism has succeeded in penetrating those lines of defence, signalling a partial or complete breakdown of the body's defence system.

Natural resistance

The body's FIRST LINE OF DEFENCE comprises the intact cell layers of skin and mucous membrane, which form a physical barrier. The skin's low pH level and bactericidal fatty acids enhance the protection provided by this physical barrier. The defences in the respiratory tract and the gastrointestinal tract are mucus, the 'ciliary elevator' of the epithelium, and the motility of the small intestine. The presence of normal microbial flora (colonisation resistance) in the intestine also plays a role in protection against colonisation by external bacteria.

The most important humoral NATURAL resistance factors are complement, ANTIMICROBIAL PEPTIDES, LYSOZYME, interferon, and a number of CYTOKINES (see chapters A5 and A6). ANTIMICROBIAL PEPTIDES are widely expressed as part of the professional phagocyte antimicrobial arsenal and rapidly induced at epithelial surfaces. They are found in mammals, invertebrates, and plants. In general, they are small, amphipathic molecules, contain positive charge and can be structurally divided into several categories. The mode of action goes beyond their antimicrobial capacities and they elicit a complex array of responses in different cell types. The most

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Table 1. Some examples of important human pathogens

	Species	Disease/location	Treatment/prevention
Bacterium	*Streptococcus pneumoniae*	Pneumoniae/meningitis	Antibiotics/vaccination
	Mycobacterium tuberculosis	Lung tuberculosis	Antibiotics
	Vibrio cholera	Severe diarrhoea	Antibiotics/liquid suppletion/ sanitation
	Staphylococcus aureus MRSA	Wound infection/hospital infection	Antibiotics, MRSA not sensitive for standard antibiotics, difficult to treat
	Neisseria meningitidis	Meningitis	Antibiotics/vaccination
	Bacillus anthracis	Systemic infection (sepsis)	Antibiotics as early as possible
	Corynebacterium diphtheriae	Throat/heart	Antiserum, vaccination
	Campylobacter jejuni	Intestinal infections	Hygiene, especially food (chicken)
	Helicobacter pylori	Gastritis, ulcer	Antibiotics
DNA Virus	Poxviridae	Smallpox	Vaccination, eradication
	Herpesviridae	Herpes genitalis	Anti-viral agents
	Papavoviridae	Warts and cervical carcinoma	Surgery
	Hepadnaviridae	Hepatitis B	Vaccination
RNA Virus	Orthomyxoviridae	Influenza	Vaccination
	Coronaviridae	SARS	Unknown
	Retroviridae	AIDS	Anti-viral agents
	Caliciviridae	Gastrointestinal infection	Sanitation, hygiene
Parasites	*Plasmodium* species	Malaria	Prophylactic medication, anti-malarial drugs
	Giardia species	Intestinal tract	Hygiene
	Trypanosoma cruzi	Sleeping sickness	Anti-parasitic agents

extensively studied mammalian gene families are the cathelicidins and DEFENSINS. In general the ANTIMICROBIAL PEPTIDES disrupt lipid membranes and thereby induce microbial killing. Micro-organisms have developed several countermeasures against ANTIMICROBIAL PEPTIDES but the many structurally different peptide classes still provide protection against infection.

LYSOZYME, which is found in almost all body fluids, degrades sections of the cell wall of Gram-positive and – in combination with complement – Gram-negative bacteria. This causes the otherwise sturdy cell wall to leak and the bacterium to burst.

Interferons are glycoproteins and may inhibit the replication of viruses. Within several hours after the onset of a virus infection, INTERFERONS are produced in the infected cell and help protect the neighbouring unaffected cells against infection. This protection is brief, but high concentrations of INTERFERONS are produced at a time when the primary immunological response is relatively ineffective.

CYTOKINES, such as INTERLEUKIN-2 (IL-2), granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumour necrosis factor-α (TNF-α), stimulate non-specifically the proliferation, maturation, and function of the cells involved in defence (see chapter A6).

Innate immune cells recognise microbes by TOLL-LIKE RECEPTORS (TLR) (see section *Pathogenesis of* SHOCK), giving rise to the above production of CYTOKINES in the early phase of the response.

Micro-organisms that succeed in penetrating the FIRST LINE OF DEFENCE are ingested, killed, and degraded by phagocytic cells [POLYMORPHONUCLEAR LEUKOCYTES (PMN) or NEUTROPHILS, MONOCYTES, and MACROPHAGES], which are attracted to a microbial infection through CHEMOTAXIS. The ingestion by phagocytic cells of the micro-organism is enhanced by serum proteins (opsonins), such as ANTIBODIES and the C3b component of complement, which are recognised by specific RECEPTORS on the PHAGOCYTES. After ingestion, the particle is surrounded by the membrane of the phagocyte, forming a vacuole known as a PHAGOSOME. The PHAGOSOME then fuses with some of the countless granules in the phagocyte, thus allowing the lysosomal microbicidal agents and enzymes to do their work. The formation of toxic oxygen radicals greatly contributes to the killing and elimination of the ingested micro-organism (Fig. 1) (see chapter A7).

A special role in cellular NATURAL resistance is reserved for the NATURAL KILLER CELLS (NK cells), which display considerable CYTOTOXIC activity against virus-infected cells. This NK activity is stimulated by INTERFERONS and, at a very early stage in the infection, serves to reinforce the non-specific defence mechanism.

Specific resistance

In the specific immune response, elements of the NATURAL defence mechanism are directed against a specific enemy. Depending on the micro-organism, either the cellular defence mechanism (tuberculosis) or the humoral ANTIBODY-dependent defence mechanism (influenza) is of primary importance. In many cases, a joint cellular and humoral response is needed to provide an effective immune defence (typhus).

Both T LYMPHOCYTES and MACROPHAGES play a role in cellular defence. During the first contact with an antigen, MACROPHAGES process the antigen and present its protein fragments (T cell EPITOPES) to T cells, which then proliferate and remain present for years in the body as memory cells. When a second encounter occurs, T cells produce lymphokines, which activate the MACROPHAGES. These activated MACROPHAGES grow larger, produce more and better degrading enzymes, and are now able to eliminate micro-organisms, which otherwise would have survived intracellularly (tuberculosis, typhoid fever). MACROPHAGES from non-immune animals are not able to eliminate these micro-organisms.

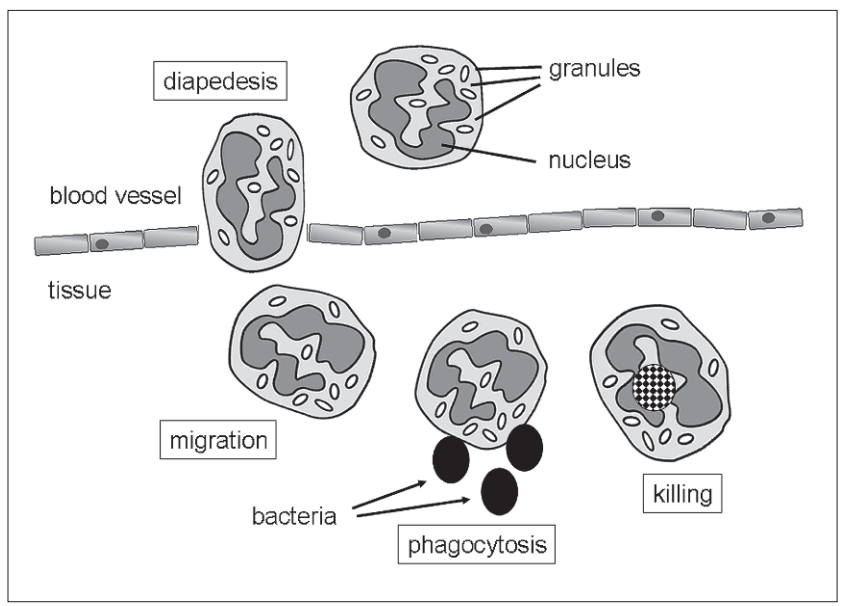

FIGURE 1
Schematic representation of the progressive steps of phagocytic endocytosis.

Five different classes of ANTIBODIES can be distinguished in man, namely, IgG, IgA, IgM, IgD, and IgE. They differ from one another in size, charge, amino acid composition, and glycosylation (see chapters A3, C2). In principle, the structure of the ANTIBODIES is the same, i.e. two heavy and two light chains: it is the variable part of these chains that recognises the micro-organism. The biological function (see below) is determined by the constant part (Fc) of the heavy chain. With the exception of IgD, all these ANTIBODIES are important in antimicrobial activity.

- IgA, which is found in all external secretions, reacts with the surface of micro-organisms, preventing them from adhering to sensitive cells and mucous membranes.
- IgG neutralises microbial toxins.
- IgG, IgM, and C3b serve as opsonins, which promote phagocytosis.
- IgG, IgM, and to a lesser extent IgA activate the complement system after binding to the micro-organism. Activation products C3a and C5a ensure that the phagocytes are attracted to the inflammatory response.
- IgG and IgM, in combination with complement and lysozyme, have a lytic effect on Gram-negative bacteria and enveloped viruses.
- IgG and IgM inhibit the mobility of micro-organisms by attaching specifically to the flagellum. Thereby the chance of phagocytosis increases and the chance of spreading of disease decreases.
- IgG, together with the killer or K cells, can eliminate infected host cells which carry viral or other foreign antigens on their surface.
- IgE is of importance in parasite infections. At the site of the infection, mast cells, bearing specific IgE, release large quantities of vasoactive amines, which cause the contraction of smooth muscle tissue and increase the permeability of the blood vessels. In the intestine, this results in worms being detached and eliminated.

Defence against bacteria, viruses, fungi, and parasites

Several non-invasive bacteria, i.e. those that do not invade the body, cause disease through the production of EXOTOXINS (tetanus, diphtheria, cholera). The IMMUNE SYSTEM neutralises the toxin with the aid of ANTIBODIES (IgG, IgM). If the individual has not been inoculated, the toxin will act on certain cells in the body directly through a RECEPTOR. This bond is very strong (i.e. has a high AFFINITY), and is difficult to break by the administration of ANTIBODIES. In practice, if there are clinical symptoms of the disease, then large doses of antitoxins must be administered. If one is trying to prevent the development of the disease, then the presence of small quantities of specific ANTIBODIES (IgG) is sufficient.

The ADHERENCE of bacteria to cells is effectively blocked by IgA. Oral vaccination against cholera, for example, is aimed at obtaining sufficient specific IgA in the intestine, so that no colonisation of this bacterium can take place, and the cholera toxin can no longer adhere to its RECEPTOR.

In general, defence against invasive bacteria is provided by ANTIBODIES (IgG, IgM) that are directed against bacterial surface ANTIGENS. In many cases, these bacteria have a CAPSULE, which interferes with effective PHAGOCYTOSIS. ANTIBODIES against the ANTIGENS of these capsules neutralise the interference, with subsequent elimination of the bacteria by PHAGOCYTES. ANTIBODIES (IgM, IgG, IgA) in combined action with complement kill bacteria by producing holes in the cell wall of the bacterium.

Although intracellular bacteria (tuberculosis, leprosy, listeriosis, brucellosis, legionellosis, and salmonellosis) are ingested by MACROPHAGES, they are able to survive and multiply. In these cases, cellular immunity alone provides the defence, since ANTIBODIES are not effective. Only activated MACROPHAGES are capable of killing and degrading these bacteria.

ANTIBODIES neutralise viruses directly and/or indirectly by destroying infected cells that carry the virus antigen on their surface. The mechanisms of this defence resemble those of humoral defence against bacterial surfaces. The ANTIBODY-dependent cellular CYTOTOXICITY reaction is specific for defence

against viruses. Cells that carry an antigen encoded by the virus on their surface are attacked by CYTOTOXIC K cells, bearing ANTIBODIES that fit the antigen on the TARGET cell (K cells have Fc RECEPTORS for IgG). (Fig. 2)

Not only humoral, but also cellular immunity plays an important role in virus infections. People with a genetic T cell deficiency are highly susceptible to virus infections. In cellular defence, it is primarily the virus-infected cells that are attacked and eliminated. CYTOTOXIC T cells recognise MHC class I-presented T cell EPITOPES on the surface of virus-infected cells and kill them.

The fungi responsible for human diseases can be divided into two major groups on the basis of their growth forms or on the type of infection they cause. Pathogens exist as branched filamentous forms or as yeasts, although some show both growth forms. The filamentous types (*Trichophyton*) form a 'mycelium'. In asexual reproduction, the fungus is dispersed by means of spores; the spores are a common cause of infection after inhalation. In yeast-like types (*Cryptococcus*), the characteristic form is the single cell, which reproduces by division or budding. Dimorphic types (*Histoplasma*) form a mycelium outside, but occur as yeast cells inside the body. *Candida* shows the reverse condition and forms a mycelium within the body.

In superficial mycoses, the fungus grows on the body surface, for example skin, hair, and nails (*Epidermophyton*, *Trichophyton*), the disease is mild, and the pathogen is spread by direct contact. In deep mycoses (*Aspergillus*, *Candida*, *Cryptococcus*, *Histoplasma*), internal organs are involved and the disease can be life-threatening and is often the result of opportunistic growth in individuals with impaired immunocompetence.

Many of the fungi that cause disease are free-living organisms and are acquired by inhalation or by entry through wounds. Some exist as part of the normal body flora (*Candida*) and are innocuous unless the body's defences are compromised in some way. The filamentous forms grow extracellularly, while yeasts can survive and multiply within phagocytic cells. NEUTROPHILS kill yeasts by means of both intra- and extracellular factors. Some yeasts (*Cryptococcus neoformans*) form a thick polysaccharide CAPSULE to prevent phagocytic uptake. In addition, many cell-wall components of yeasts cause suppression of cell-mediated immune responses. The role of humoral and cellular immunity in controlling infections caused by fungi is not yet well defined, but cellular immunity is the cornerstone of host defence against (some) fungal infections. As a consequence, HIV INFECTION, which affects the cellular arm of the IMMUNE SYSTEM, results in previously uncommon infections such as those caused by *C. neoformans*.

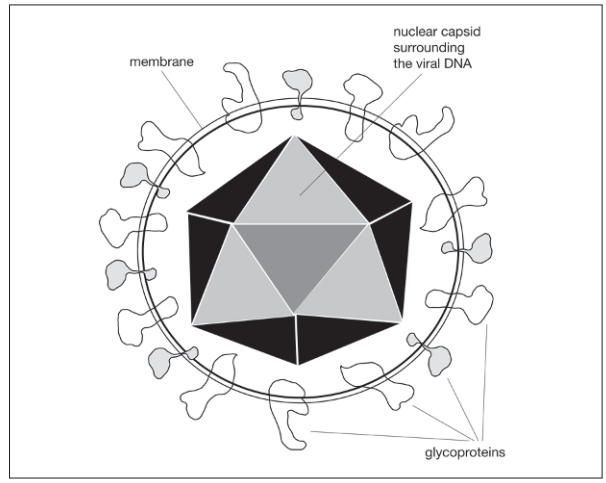

FIGURE 2
*Schematic illustration of an enveloped virus (*Herpes simplex *virus).*

The immunological defence systems against *parasites* are considerably more complex than those against bacteria and viruses. This is due to various factors. In the first place, each parasite has its own life cycle, consisting of various stages with specific antigen compositions. Moreover, parasites are able to avoid the host defence system (mimicry), to combat it (IMMUNOSUPPRESSION), or to mislead it (antigenic variation). Both humoral and cellular immunity are important for the defence against parasites growing intercellularly, as we have seen in the case of bacteria and viruses. ANTIBODY concentrations (IgM, IgG, IgE) are often elevated. IgE also plays a special role in the removal of parasites (especially worm infections) from the intestine (see above).

Table 2. Classification of micro-organisms

Classification of bacteria by: **Genotypical characteristics**: chromosomal DNA fragment analysis, nucleic acid sequence analysis, probes **Phenotypical characteristics**: morphology, biotyping, serotyping, antibiotic resistance **Analytical characteristics**: cell-wall analysis, lipid and protein analysis, enzyme typing (catalase) **Gram staining positive or negative** **Aerobic, anaerobic**: Fermentation of different sugars
Naming and classification of viruses according to: **Structure**: size, morphology (naked, enveloped), nucleic acid (RNA, DNA) **Molecular aspects**: mode of replication, assembly and budding **Disease**: encephalitis, hepatitis **Means of transmission**: droplets, water, blood, insects **Host range**: animal, plant, bacteria
Classification of fungi according to: **Structure**: macroscopic morphology of hyphae (mycelium); microscopic morphology of hyphae, conidophores and conidia (spores); and shape and size **Cell features**: nucleus, cytosol, plasmalemma (cell membrane which contains cholesterol), physiology, staining properties **Sexual characteristics**: sexual and /or asexual reproduction, extended dikaryotic phase, basidium formation **Genotypical characteristics**: chromosomal DNA fragment analysis, nucleic acid sequence analysis, probes
Diagnosis of parasites by: **Macroscopical examination** **Concentration of cysts and eggs by microscopic examination** **Serological diagnosis**: antibody response **Detection of parasite by serology and by nucleic acid hybridisation**: probes and amplification techniques

Pathogenesis of shock

Sepsis is a systemic inflammatory response to presumed or known infection. The resulting inflammatory response becomes over amplified, leading to multiple organ failure and death. Pathogen-associated molecular patterns (PAMP) in bacteria, viruses, parasites, and fungi initiate the host response by triggering families of pattern recognition receptors (PRR). In Gram-negative (Fig. 3) bacterial infections, the interaction between bacterial endotoxin or lipopolysaccharide (LPS; a major structural component of the cell wall) and various host-cell systems has been implicated in the pathogenesis of septic shock. In particular, the release of TNF-α and interleukin-1 (IL-1) after the activation of host cells by endotoxin induces haemodynamic shock.

Biochemical and genetic evidence has identified TLR4 as the receptor that mediates cellular activation in response to LPS. This family of TLR proteins (Fig. 4), which resemble the antimicrobial Toll proteins of *Drosophila* (fruit fly), has been identified in humans and mice. TLR4 was identified as the missing link in LPS-induced cell signal transduction and responsiveness that is associated with MD-2 and CD14. It is known that C3H/HeJ mice are hyporesponsive to the biological effects of LPS. This proved to be the result of TLR4 deficiency. The TLR family members are coupled to a signalling adapter protein (MyD88) and form differential dimers that may explain the discrete responses to TLR ligands such as lipoproteins, heat shock proteins, unmethylated CpG DNA, viral dsRNA and bacterial flagellin. Intracellular signalling involves several kinases depending on

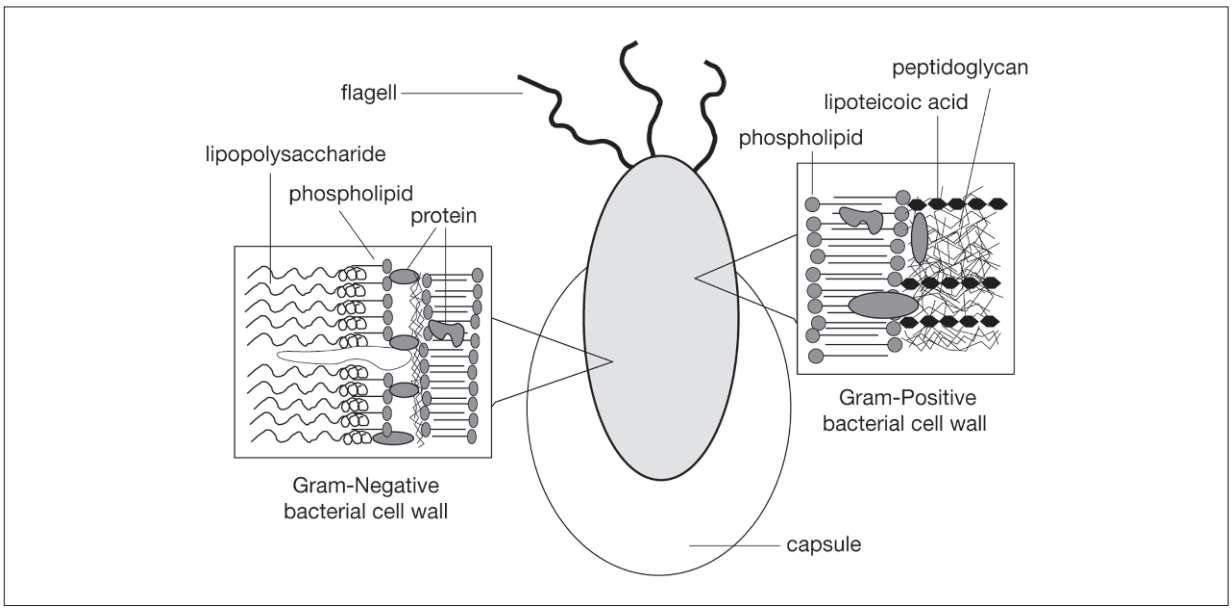

FIGURE 3
Schematic illustration of the cell envelope of a Gram-negative and a Gram-positive bacterium.

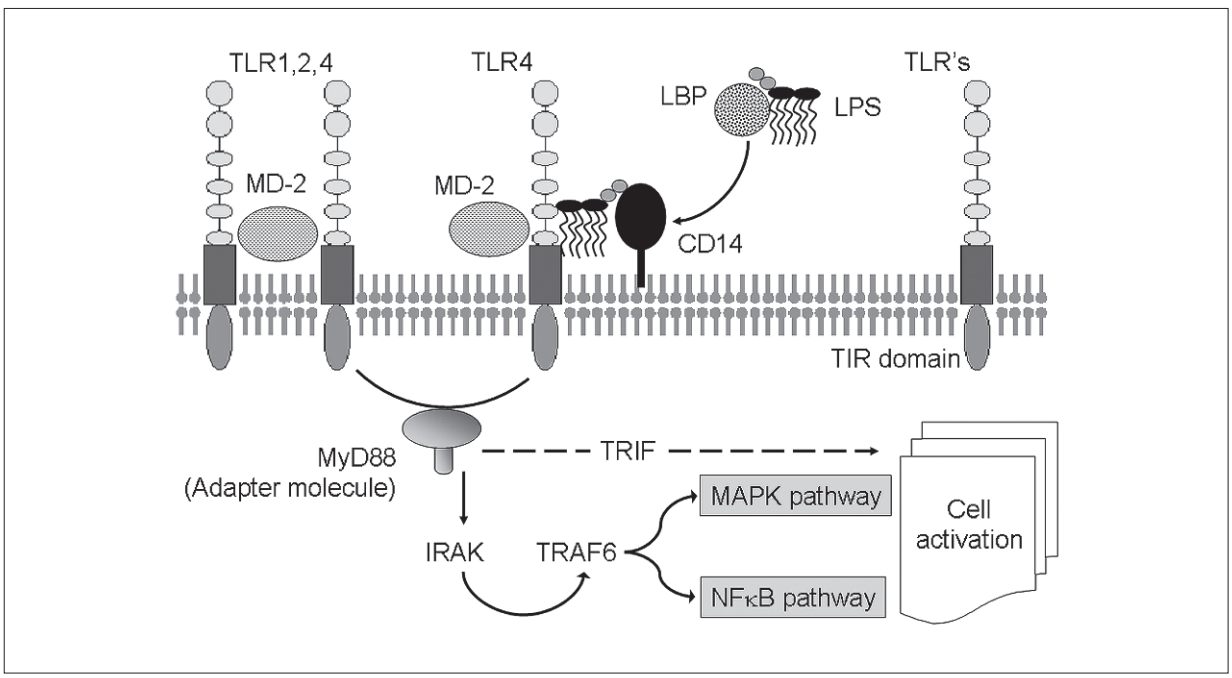

FIGURE 4
Schematic illustration of cell activation through Toll-like receptors (TLRs).

the TLR involved and includes the MAP kinase and NF-κB pathways leading to a cellular response. PAMP for TLR2 include a variety of agonists derived from Gram-positive organisms such as PEPTIDOGLYCAN and lipoteichoic acid. Therefore, it seems that TLR2 and TLR4 are activated primarily by different PAMP to initiate the host response to Gram-positive and Gram-negative bacterial infection, respectively. This is further illustrated by the fact that TLR2 (but not TLR4) knockout mice are highly susceptible to GRAM-POSITIVE BACTERIA, like *Staphylococcus aureus*, whereas TLR4 (but not TLR2) knockout mice are highly susceptible to Gram-negative bacteria such as *Salmonella typhimurium*.

Several lines of evidence support the current hypothesis that the monocyte/macrophage is the principal cellular mediator of endotoxicity. LPS-hyporesponsive TLR4-deficient C3H/HeJ mice are made responsive by transfer of MACROPHAGES of a closely related LPS-sensitive strain. When the host is challenged with LPS, soluble factors are produced by MACROPHAGES that mediate fever and an acute-phase response. These factors include the pro-INFLAMMATORY CYTOKINES, IL-1, IL-6, IL-8, and TNF-α. Together, TNF-α and IL-1 stimulate endothelial cells to produce and express proteins on their membrane that have adhesive properties for LEUKOCYTES, promoting the margination and passage of neutrophils from blood vessels through the endothelial layer, leading to neutrophil influx into the tissue. ADHESION MOLECULES that mediate the binding of neutrophils appear on the endothelium after an inflammatory stimulus, followed by molecules that are specific for adhesion of MONOCYTES or LYMPHOCYTES, which may be why NEUTROPHILS enter before mononuclear cells. Molecules that are currently known to be involved in leukocyte-endothelium interactions belong to three structural groups: the immunoglobulin gene superfamily, the integrin family, and the selectin family.

Concomitant with cytokine release, LPS induces the activation of neutrophils, MACROPHAGES, and many other cells, resulting in the release of toxic oxygen radicals, which lead to tissue damage. At the same time, membrane-associated phospholipases are activated and products of the arachidonic acid cascade are released through the CYCLOOXYGENASE and/or LIPOXYGENASE pathways (see chapter A7). PLATELET-ACTIVATING FACTOR (PAF) is also generated, partly in response to the same signals. All these products contribute to a generalised inflammatory state with influx of neutrophils, capillary-leak syndrome, disturbances in blood coagulation, and myocardial suppression.

ENDOTOXIN and TNF-α also trigger multiple abnormalities in coagulation and fibrinolysis, leading to microvascular clotting and diffuse intravascular coagulation. They also induce endothelial cells to produce plasminogen activator and IL-6, which is an important modulator of the production of acute-phase proteins by the liver. Interestingly, despite having important structural differences, TNF-α and IL-1 have multiple overlapping and few distinct biological activities, act synergistically, and mimic the whole spectrum of toxicity caused by LPS (see chapter A5). IL-8 is an important chemoattractant and activator of NEUTROPHILS and is crucial in the early stages of INFLAMMATION.

Infusion of ENDOTOXIN in healthy humans leads to an early and transient increase in plasma levels of TNF-α (detectable after 30 minutes, peaking after 90–120 minutes, and undetectable after 4–6 hours), which coincides with the development of clinical symptoms and pathophysiological responses encountered in Gram-negative septicaemia. TNF-α, IL-1, IL-6, and IL-8 levels are also increased in patients with sepsis syndrome, with high levels of these CYTOKINES being correlated with severity of disease.

All these observations support the concept that ENDOTOXIN largely acts by initiating an inflammatory response through the activation of MONOCYTES/MACROPHAGES and the subsequent release of CYTOKINES. It also activates the COMPLEMENT SYSTEM (leading to the generation of C5a, which induces aggregation of neutrophils and pulmonary vasoconstriction) and factor XII of the intrinsic coagulation pathway (Hageman factor). Finally, it induces the release of ENDORPHINS, which are also involved in the complex interactions of the inflammatory response in endotoxic septic SHOCK.

GRAM-POSITIVE BACTERIA are frequently and increasingly cultured from blood obtained from patients in SHOCK. Unlike the pathophysiology of SHOCK caused by Gram-negative bacteria, not much is known about the sequence of events that controls the signalling of MONOCYTES and MACROPHAGES that leads

to the release of CYTOKINES. Cell-wall components, such as PEPTIDOGLYCAN and teichoic acid, are clearly important in the activation of these cells. EXOTOXINS, however, may also play a role in the pathogenesis of Gram-positive bacterial SHOCK.

Recently, another protein family was identified that also participates by sensoring microbial components derived from bacterial PEPTIDOGLYCAN. The NOD (NUCLEOTIDE-BINDING OLIGOMERISATION DOMAIN) proteins NOD1 and NOD2 have that have been implicated in intracellular recognition of the core structure, γ-D-glutamyl-meso-diaminopimelic acid, present in PEPTIDOGLYCAN.

A number of circulating inflammatory mediators have been investigated as marker tools to facilitate the early recognition of sepsis. These include IL-1, IL-6, TNF, pro-calcitonin, and triggering RECEPTOR on myeloid cells (TREM-1). TREM-1 is expressed on LEUKOCYTES and TREM family members have been implicated in mounting the inflammatory response. Pro-calcitonin (PCT) and IL-6 have proved to be the most prominent biomarkers of early sepsis. PCT is the pro-hormone of the hormone calcitonin, and can be produced by several cell types and many organs in response to pro-inflammatory stimuli, in particular by bacterial products. More recently HIGH MOBILITY GROUP BOX-1 (HMGB-1) has been implicated as a lethal mediator of systemic INFLAMMATION. HMGB-1 is a nuclear and cytosolic protein widely studied as a transcription and growth factor that is released into the extracellular environment. It has a weak pro-inflammatory activity by itself and it may work in concert with other pro-INFLAMMATORY CYTOKINES. This molecule may also be useful as a biomarker in the stratification of sepsis.

Susceptibility to sepsis can be influenced by factors that include ethnicity, gender, age, genetic defects and environmental factors. Single-nucleotide polymorphisms (involving single base-pair alterations) have been described in genes controlling the host response to infection such as alterations in TNF RECEPTORS, IL-1 RECEPTORS, coagulation factors and TLR. It is now clear that sepsis is a complex, dynamic syndrome with great heterogeneity, and not a distinct disease. Therefore, neutralisation of a single key mediator as a cure for all patients with sepsis is erroneous.

Human immunodeficiency virus infection

The HIV is a retrovirus that infects cells bearing the CD4 antigen, such as T helper cells (Th), MACROPHAGES, and DENDRITIC CELLS. The CD4 molecule, together with other RECEPTOR molecules, like chemokine RECEPTOR CCR5, acts as a binding site for the gp120 envelope glycoprotein of the virus. In an attempt to respond to HIV ANTIGENS and concomitant secondary microbial infections, these cells are activated, thus inducing the replication of HIV in the infected CD4 T cells, which are finally destroyed. In contrast, HIV-1 infection of MACROPHAGES is self-sustained and results in an inexorable growth of chronic active inflammatory processes in many tissue compartments including the CENTRAL NERVOUS SYSTEM. Infected cells bear the fusion protein gp41 and may therefore fuse with other infected cells. This helps the virus to spread and accounts for the multinucleated cells seen in lymph nodes and brain. As a result of the decreased numbers of $CD4^+$ Th cells and defects in antigen presentation, depressed immune responses in these patients are observed. During the progression of the disease, OPPORTUNISTIC INFECTIONS by otherwise harmless micro-organisms can occur. These include *Candida albicans* oesophagitis, mucocutaneous herpes simplex, toxoplasma in the CENTRAL NERVOUS SYSTEM, and pneumonia caused by toxoplasma and *Pneumocystis carinii*; Kaposi's sarcoma also occurs frequently in these patients. This has been linked to the presence of a previously unknown type of herpes virus (HHV-8). This immune deficiency syndrome is called 'acquired immune deficiency syndrome' (AIDS). It has been suggested that infected MONOCYTES/MACROPHAGES carry the HIV virus into the brain where it replicates in microglia and infiltrating MACROPHAGES. As a consequence, many AIDS patients develop cognitive and motor brain impairments. However, the picture is complicated by the various persistent infections already present in these patients, which give rise to their own pathology in the brain. These include *Toxoplasma gondii*, *Cryptococcus neoformans* and JC virus.

So far, no cure for HIV INFECTION has been achieved. The main effort in the prevention of HIV INFECTION lies

in mass public education programmes. Treatment of infected individuals is possible but expensive. HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) reduces morbidity and mortality among patients infected with HIV (see Fig. 5). Success is limited by the emergence of drug-resistance viruses that can be transmitted to newly infected individuals. Resistance, drug toxicity, and poor patient ADHERENCE lead to treatment failure and necessitate continuous development of alternative treatment strategies that intervene with the HIV replication cycle: i.e. on the level of virus entry, critical viral enzymes [reverse transcriptase (RT), integrase (IN) and proteases (PR)] or viral nucleocapsid (NC) protein. At this moment a triple therapy is being prescribed in Western countries (two RT inhibitors and one PR inhibitor, Fig. 4), each of which interfere with specific steps in the process of HIV replication. One major problem that has arisen is the increasing resistance to these drugs. Agents with novel MECHANISMS OF ACTION provide options for patients with DRUG-RESISTANT virus. Blocking of the chemokine RECEPTOR CCR5, a co-RECEPTOR on CD4 cells for HIV, is an alternative treatment for persons infected with the R5 HIV type. This notion is supported by a recent finding that a homozygous defect in this chemokine RECEPTOR accounts for resistance of multiple-exposed individuals to HIV-1 infection. Currently a commercially available drug is being used that specifically binds to CCR5 on the surface of the CD4 cell and selectively blocks HIV-1 binding.

Vaccines and vaccination

Pasteur and Koch triggered the stormy development of vaccines (anthrax, rabies, cholera) at the end of the 19th century. While Pasteur remained faithful to the principle of attenuated micro-organisms in preparing his vaccines, Koch employed killed germs (cholera) as a vaccine. Since diphtheria and tetanus cause disease by means of toxins, the next logical step in the development of vaccines was the use of detoxified toxins to induce protection against these diseases [diphtheria (Von Behring) and tetanus (Kitasato)]. Von Behring and Kitasato were the first to demonstrate that the source of the protective activity induced by vaccines was present in blood serum. Von Behring was also the first to prove that protective immunity could be passed on *via* serum. The development of new vaccines had its ups (yellow fever) and downs (tuberculosis). With the arrival

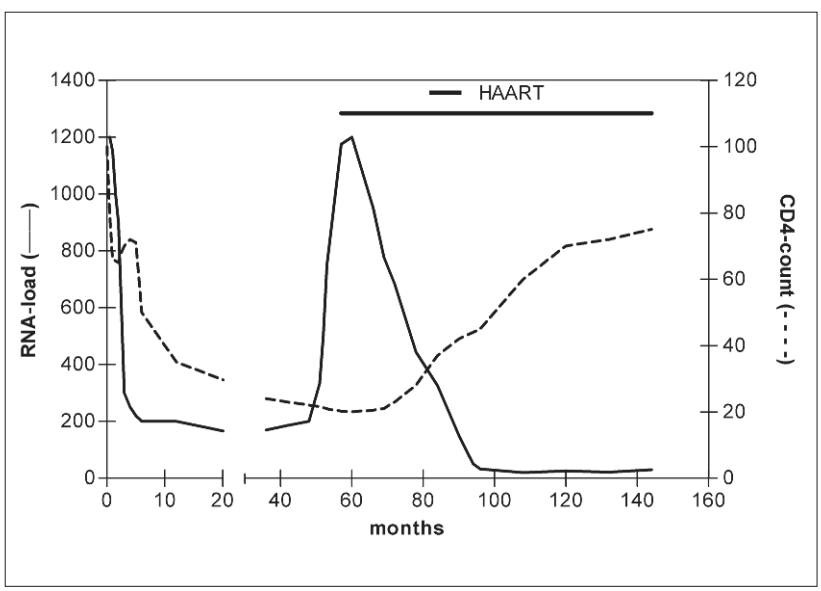

FIGURE 5
The effect of single and triple therapy on viral load and CD4 cells over time in HIV-infected individuals.

of antibiotics, all work on new bacterial vaccines was suspended or severely curtailed, although some researchers continued to work on viral vaccines, such as rubella, measles, polio, and mumps.

Since it has proved difficult to consistently develop new antibiotics to combat antibiotic-resistant bacteria, interest in vaccines has gradually increased over the last 15 years (see chapter C1). Today, thanks to new insights into the IMMUNE SYSTEM and modern molecular biological and chemical techniques of analysis and synthesis, it is possible to produce well-defined vaccines. These contain only those determinants of the PATHOGENIC MICRO-ORGANISM that induce protection (EPITOPES). These EPITOPES are usually short peptide or oligosaccharide chains, which can be produced synthetically or by means of RECOMBINANT DNA techniques. The immunogenicity of these products can be enhanced by coupling them to a CARRIER (tetanus toxoid, liposomes) and/or by adding an adjuvant (a substance that strengthens the immune response non-specifically). The RECOMBINANT DNA technique can also be used to obtain attenuated strains of micro-organisms, which are fully immunogenic and thus provide protection, but which are no longer virulent. One example of this is the development of a new cholera vaccine based on a bacterium that has all the characteristics of a virulent strain, except the toxin. The bacterium has retained all its ADHERENCE factors, which allow it to adhere to the intestinal mucosa; the length of time it spends in the intestine is sufficient to stimulate the local IMMUNE SYSTEM. The newest trend in vaccinology is immunisation by introducing plasmid DNA into the host. Success has been attained by this method for hepatitis B vaccination.

Not only are new vaccines being developed, but it is also possible to heighten NATURAL resistance for longer or shorter periods. Various INTERLEUKINS (IL-2, GM-CSF) and INTERFERONS are being studied in order to use them to combat infectious diseases (see chapter C7). Passive ANTIBODY therapy with polyclonal or MONOCLONAL ANTIBODIES (mouse or human IgG with single SPECIFICITY) for infectious diseases is experiencing a renewed interest. Targeting soluble factors (like neutralisation of bacterial toxins and viruses) or common structures (like bacterial adhesins or viral entry factors) in high-risk patient groups may be beneficial alone or may enhance the therapeutic EFFICACY of other drugs. Targets for clinical development of MONOCLONAL ANTIBODIES include multi-resistant staphylococci and enterococci, *Bacillus anthracis* toxin, HIV, HEPATITIS C VIRUS (HCV), and respiratory syncytial virus (RSV).

Infections in the new millennium

As outlined above for a number of bacteria and viruses, effective vaccines have been developed and applied worldwide. The eradication of smallpox (*Variola major*) virus in the 1970s was a milestone for the World Health Organisation. The next goal of the WHO is to eradicate poliovirus in the coming years. Major problems to be dealt with are the distribution of these vaccines, the costs involved, the registration and the compliance of the vaccinees and molecular techniques to trace the final bug. Meanwhile new unexpected microbiological threats become in focus. Hospital infections caused by multi-resistant micro-organism due to the abundance use of antibiotics and exchange of genetic material between micro-organisms impose major problems on patients and healthcare workers. New antibiotics and/or vaccines should be developed and new strategies employed to contain these infections. Due to crowding and the high mobility of the world population, old and new pathogens, e.g. influenza and SARS, threaten our society. The recent influenza A H1N1 ("Mexican") flu outbreak in 2009 demonstrated how rapidly a new strain of flu can emerge and spread around the world. The sudden outbreak of this novel flu virus has tested the world's public health preparedness. In the Netherlands, Q fever emerged with large epidemic outbreaks. Q fever is a zoonotic disease, that is passed from infected (farm) animals (cattle, sheep, and goats are the primary carriers) to humans. Q fever is a disease caused by the intracellular bacterium *Coxiella burnetii*. Organisms are excreted in milk, urine, and faeces of infected animals. Most importantly, during birthing the organisms are shed in high numbers within the amniotic fluids and the placenta. *C. burnetii* is resistant to heat, drying, and many common disinfectants, allowing it

to survive for a long time in the environment. People can become infected by inhalation of the bacteria, but the risk of infection is low. Only about one-half of all people infected with *C. burnetii* show signs of clinical illness (e.g. flu-like illness, pneumonia, and hepatitis). Q fever can be treated with antibiotics, and most people will recover fully. As a control measurement pregnant goats on dairy farms had to be killed and a mandatory vaccination campaign was started.

On top of this, terrorists might intentionally use micro-organisms (smallpox, anthrax, plague etc.), or bacterial toxins (botulism) to cause death and disease in humans or animals in a civilian setting. The recognition that an event was caused by a biological weapon presents a severe challenge to be prepared for such an attack, especially for medical care providers, and public health officials. Strategies to combat bioterrorism have to be worked out but with the experience of 100 years of combating micro-organisms with hygiene measures, vaccination, antibiotic and anti-viral treatment, there must be a way out.

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Important websites

Scientific Research

American Society for Microbiologists, *http://www.asm.org*

American Association of Immunology, *http://www.aai.org*

European Federation of Immunological Societies, *http://www.efis.org*

Federation of European Microbiological Societies, *http://www.fems-microbiology.org*

National Library of Medicine, *http://www.ncbi.nlm.nih.gov*

Outbreaks of Infectious Diseases

Centers for Disease Control and Prevention, *http://www.cdc.gov*

International Society for Infectious Diseases, *http://www.isid.org*

Daily update: *http://www.promedmail.org*

World Health Organisation, *http://www.who.int*

Immune response in human pathology: Hypersensitivity and autoimmunity

Jacques Descotes

Introduction

The IMMUNE SYSTEM is a complex network of effector/regulatory cells and molecules whose primary function is to maintain homeostasis by discriminating self from non-self. Closely interacting processes ensure co-ordinated immune responses. The differentiation, activation, and renewal of specialized cells are required to achieve adequate immune responsiveness under the control of many mechanisms with either compensatory or conflicting outcome. It is noteworthy, however, that immune responses are not always beneficial. Thus, inadvertent immunological reactivity against "innocent" ANTIGENS can lead to HYPERSENSITIVITY reactions, while immune responses against self constituents of the host can result in AUTOIMMUNITY. Although either adverse immune response can be inhibited, at least to some extent, by pharmacological manipulation, drugs as well as environmental or industrial chemicals can trigger both HYPERSENSITIVITY and autoimmune reactions [1].

Hypersensitivity

Nearly every chemical in our medical, domestic, occupational or NATURAL environment has the potential to induce HYPERSENSITIVITY reactions. Although HYPERSENSITIVITY is widely considered to a major cause of immunotoxic events, reliable data on the overall incidence of drug-induced HYPERSENSITIVITY reactions in human beings are lacking [2]. Indeed, most data published so far have consisted of isolated case reports, or results from studies focusing on selected groups of patients, clinical manifestations, risk factors or causative drugs. HYPERSENSITIVITY reactions might account for 10% of all drug-induced adverse events, but this is merely a rough estimate based on the author's experience. Similarly, any estimate of the incidence of HYPERSENSITIVITY reactions in relation to occupational or environmental exposures as well as food allergies is deemed to be fraught with inaccuracy.

Clinical manifestations of drug-induced hypersensitivity reactions

Clinically, HYPERSENSITIVITY reactions are extremely varied as nearly every organ or tissue of the body can be affected, even though one organ or tissue is often a predominant TARGET in a given reaction [3].

Anaphylactic shock

Anaphylactic SHOCK is a life-threatening reaction with an estimated death rate of less than 1%, so that deaths from anaphylactic SHOCK in the general population may account for less than one per million [4]. ANAPHYLAXIS develops shortly – within the first 2–4 hours in the vast majority of cases – after the last contact with antigen, the shortest delay being typically associated with the intravenous route. Patients complain of itching, urticaria and/or angioedema as well as tachycardia and hypotension progressing to cardiovascular collapse or SHOCK in the most severe cases, and marked respiratory difficulties with cyanosis [5, 6]. Anaphylactic SHOCK is a major medical emergency. Initial supportive measures tend to maintain or restore normal respiratory and circulatory functions. One key treatment is adrenaline (epinephrine) injected subcutaneously, intramuscularly, or intravenously in cases of imminent death [7].

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Skin reactions

Skin reactions are the most frequent immune-mediated adverse effects of drugs. Drug-specific IgE ANTIBODIES leading to urticaria or angioedema, or circulating immune complexes possibly associated with vasculitis are rather uncommon, so that immune-mediated cutaneous manifestations caused by drugs are thought to be primarily T cell mediated [8, 9]. The clinical presentation is extremely varied. Morbilliform or exanthematous rash may occur in up to 2% of treated patients. Most often, the onset is within 1–2 weeks after starting treatment, but a delayed onset is possible. T cell-mediated CYTOTOXICITY is the most likely mechanism. Contact dermatitis is a frequent complication of occupational and environmental exposures, but it can also develop following TOPICAL drug applications. It is characterized by pruritic vesicles on an erythematous background [10]. Allergic contact dermatitis is a T LYMPHOCYTE-mediated reaction that should be differentiated from irritant contact dermatitis caused by non antigen-specific mechanism(s) [11]. Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are the most severe and potentially life-threatening cutaneous complications of drug treatments [12–14]. They may affect 1 to 6 in 1 million persons per year; 80% of TEN are considered to be drug-induced with a lower incidence for SJS. Typically, the first clinical symptoms, i.e. flu-like reaction of variable severity and mucous membrane involvement, are observed 7–21 days after the start of treatment. Skin lesions expand within 2–3 days with purpuric macules and bullae leading to erosion of the epidermis. Painful erosions of the mucous membranes account for dysphagia, conjunctivitis, keratitis, diarrhea and/or respiratory distress. The prognosis and management are similar to those of severely burnt patients. The mortality rate is about 5% for SJS and 30% for TEN.

The drug HYPERSENSITIVITY syndrome (DHS) or – drug reaction with eosinophilia and systemic symptoms (DRESS) – as it is nowadays often called, is characterized by fever and rash, but 50% of patients also present with lymphadenopathy, arthritis or hepatitis, and less frequently kidney, heart, lung, thyroid, or brain involvement [15]. High eosinophil count (eosinophilia) is a common finding.

Immunoallergic cytopenias

IMMUNOALLERGIC cytopenias manifest as ANTIBODY-mediated destruction of one or several blood cell lines. Even though drugs have been suggested to account for 20–40% of cytopenias, the overall incidence is actually low [16]. Patients with agranulocytosis are either asymptomatic, so that diagnosis is often made after routine blood examination, or they develop clinical symptoms of infection, in particular sore throat. In this latter case, NEUTROPENIA is usually below 100/mm^3. Hemolytic anemias are due either to direct or ANTIBODY-mediated toxicity to the membrane of ERYTHROCYTES [17, 18]. Depending on whether AUTOANTIBODIES are involved or not, immune-mediated hemolytic anemias are either autoimmune or IMMUNOALLERGIC. However, the distinction may be difficult to make as one given drug can induce both AUTOANTIBODIES and drug-dependent ANTIBODIES. Most IMMUNOALLERGIC hemolytic anemias are acute or sub-acute. Clinical symptoms develop within hours following drug intake and include abdominal and dorsal pain, headache, malaise, fever, nausea and vomiting. SHOCK and acute renal failure are noted in 30–50% of cases.

Two main mechanisms have been shown to be involved. The causative drug, e.g., a third-generation cephalosporin [19], can non-specifically bind to ERYTHROCYTES and react with circulating specific ANTIBODIES. Otherwise, the drug bound to a plasma protein can form an antigenic complex with the resulting production of IgM or IgG ANTIBODIES against the drug-protein complex that can be passively fixed to ERYTHROCYTES. The reintroduction of even a tiny amount of the drug can trigger an antigen-ANTIBODY reaction leading to intravascular hemolysis by activation of the complement cascade. Drug-induced IMMUNOALLERGIC thrombocytopenias are uncommon with the notable exception of those induced by heparin [20]. Most patients with heparin-induced immune thrombocytopenia have detectable ANTIBODIES against the platelet glycoproteins Ib/IX and IIb/IIIa. Clinically, thrombocytopenia leads to bleeding when platelet counts are less than 10 000–30 000/mm^3.

Other clinical manifestations

Other clinical manifestations of drug-induced HYPERSENSITIVITY reactions include hepatitis, nephritis and pneumonitis. Severe drug-induced hepatitis is relatively infrequent and considered to be an IDIOSYNCRATIC reaction in most instances [21]. An unpredictable adverse immune response against the liver with cytolytic, cholestatic or mixed clinical and biological features, however, can be involved [22]. The exact causative mechanism is not known in most cases of IMMUNOALLERGIC hepatitis, and the association of liver injury with fever, rash and eosinophilia usually developing within 1–8 weeks after starting drug treatment is often considered to be suggestive of an IMMUNOALLERGIC reaction.

Drugs are the leading cause of acute interstitial nephritis; however, drug-induced IMMUNOALLERGIC interstitial nephritis is a rare condition nowadays [23]. The clinical presentation is non-oliguric renal dysfunction associated with fever, rash and/or eosinophilia in most patients. Drugs can also cause acute interstitial or eosinophilic pneumonia, and HYPERSENSITIVITY pneumonitis [24]. Clinical manifestations including fever, cough, eosinophilia, and elevated serum IgE levels vary depending on the offending drug.

Mechanisms of drug-induced hypersensitivity reactions

Immune-mediated HYPERSENSITIVITY reactions are the consequence of the exquisite capacity of the IMMUNE SYSTEM to recognize structural elements of non-self molecules or ANTIGENS, and to mount a specific response due to the involvement of immunological memory.

Sensitization

An absolute prerequisite for any antigen-specific HYPERSENSITIVITY reaction to develop is that SENSITIZATION has occurred prior to the eliciting contact. It is normally impossible to demonstrate whether a prior contact was sensitizing. Therefore, a prior contact can only be assumed to be sensitizing. Identifying a prior contact is more or less easy. This can be straightforward when the patient who developed an adverse reaction has been previously treated with the same drug. A minimum of 5–7 days of treatment is deemed to be necessary for SENSITIZATION to occur in patients without a prior contact. In the majority of cases, IMMUNOALLERGIC drug-induced HYPERSENSITIVITY reactions develop within the first month of treatment. A prior contact may be far less easy to detect when it is related to exposure *via* the food chain or a closely related molecule leading to cross-allergenicity. It is crucial to bear in mind that even though a prior contact is absolutely required, an adverse reaction may not necessarily develop after the subsequent contact so that drug rechallenge may be misleadingly negative.

Broadly speaking, a molecule can be suspected to be sensitizing if it is foreign and sufficiently large. The vast majority of drugs are foreign molecules. A size of approximately 10 kDa is often accepted as the minimal size for foreign molecules to be potentially immunogenic (sensitizing). Additional factors, such as structural complexity, degree of polymerization, or biodegradability play a major role [25]. As the vast majority of drugs are too small to be direct immunogens, low-molecular-weight molecules are assumed to induce SENSITIZATION by playing the role of HAPTENS [26]. HAPTENS are small molecules that strongly bind to CARRIER macromolecules so that the formed hapten-CARRIER complex can trigger a specific immune response. Sufficient chemical reactivity is absolutely required for low-molecular-weight molecules to become HAPTENS. In contrast to industrial chemicals, most pharmaceutical molecules are devoid of significant chemical reactivity, so that metabolites are postulated to be involved [27]. However, highly reactive metabolites are short-lived so that they often cannot be identified. The consequence is that evidence for the role of metabolites in drug-induced HYPERSENSITIVITY reactions is often at best indirect.

The hapten theory has been increasingly challenged over the last decade. The danger hypothesis states that the primary driving force of the IMMUNE SYSTEM is to protect against danger. Thus, when an antigen is presented to T cells, an additional signal is required for an adverse reaction to develop instead of TOLERANCE. Damage induced by reactive metabo-

lites [28], or a latent or ongoing viral infection can serve as the second signal [29]. Finally, studies with drug-specific human T cell CLONES from patients with a history of HYPERSENSITIVITY reaction evidenced a direct interaction of even poorly reactive native drugs with the T CELL RECEPTOR resulting in the activation T LYMPHOCYTES with subsequent cytokine production and CYTOTOXICITY. According to this p-i concept, the adverse reaction may occur even on the first encounter with the drug [30].

Risk factors

Even potent sensitizing drugs, such as penicillin G, only induce HYPERSENSITIVITY reactions in quite a small percentage of treated patients. The involvement of risk factors is logically assumed to account for this finding. Risk factors may be related either to the patient or to the drug. Age, gender, atopy and genetic predisposition are the main risk factors related to the patient. Young adults develop more frequent HYPERSENSITIVITY reactions to drugs for unknown reasons. An epidemiological study of severe anaphylactic and anaphylactoid reactions in hospitalized patients found 927 reactions per 1 million in patients less than 20 years of age, 221–276 in patients between 20 and 59 years of age, and only 154 in patients over 60 [31]. Young girls and women seem to develop only slightly more HYPERSENSITIVITY reactions induced by drugs than boys and men. Atopy is characterized by excessive production of IgE ANTIBODIES associated or not with one or several diseases, such as reaginic ASTHMA, hay fever and constitutional dermatitis. Due to variable definitions over time, conflicting results have been published, and it remains to be clearly established whether atopy is a risk factor in drug-induced HYPERSENSITIVITY reactions. Multi-generation family and twin studies have demonstrated a genetic component in a number of allergic diseases, in particular IgE-mediated diseases.

Limited evidence supports the role of genetic predisposition in drug-induced HYPERSENSITIVITY reactions. However, as metabolites are likely to play a pivotal role in SENSITIZATION to drugs, the genetic POLYMORPHISM of metabolic pathways involved in drug biotransformation can be suspected to be involved [32]. One example is abacavir-induced DHS, which has been reported in 4–8% of treated HIV patients in whom a strong association between the MHC class I allele HLA-B*5701 and the occurrence of DHS could be identified [33].

Major risk factors related to the drug include the chemical structure, route of administration, and treatment schedule. Although the role of specific elements of the chemical structure is suspected, much remains to be done to define reliable structure-immunogenicity relationships. Every route of administration can result in SENSITIZATION, but the TOPICAL route has a greater potential. The oral route normally leads to TOLERANCE, but the mechanism of TOLERANCE breakdown is poorly understood. In sensitized patients, the intravenous route is associated with more rapidly developing and more severe reactions. Finally, intermittent treatment regimens facilitate SENSITIZATION.

Pathophysiological mechanisms of drug-induced hypersensitivity reactions

In the early 1960s, Gell and Coombs proposed a classification of IMMUNOALLERGIC reactions, which was subsequently extended to drugs. This antique classification is still widely used, although this can often be misleading as not all mechanisms are covered, several mechanisms can be involved concomitantly, or distinct mechanisms can be involved in different patients treated with the same drug despite clinically similar reactions. Despite obvious major flaws, this classification into four types can nevertheless serve as a mere introduction to the pathophysiology of drug-induced HYPERSENSITIVITY reactions (Fig. 1).

Immediate HYPERSENSITIVITY (type I) reactions involve the production of reaginic ANTIBODIES, namely IgE and to a lesser extent IgG_4 in man, induced by the causative ANTIGENS (ALLERGENS). IgE bind to high-AFFINITY RECEPTORS (FcεRI or CD64) on the cell membrane of MAST CELLS and BASOPHILS. After a subsequent contact, but not mandatorily the next contact, the interaction between a divalent allergen and specific IgE triggers the DEGRANULATION of MAST CELLS and BASOPHILS, which results in the immediate release of preformed mediators including HISTAMINE, neutral proteases (e.g. tryptase, cathepsin D, chymase), and heparin, which are stored within cytoplasmic

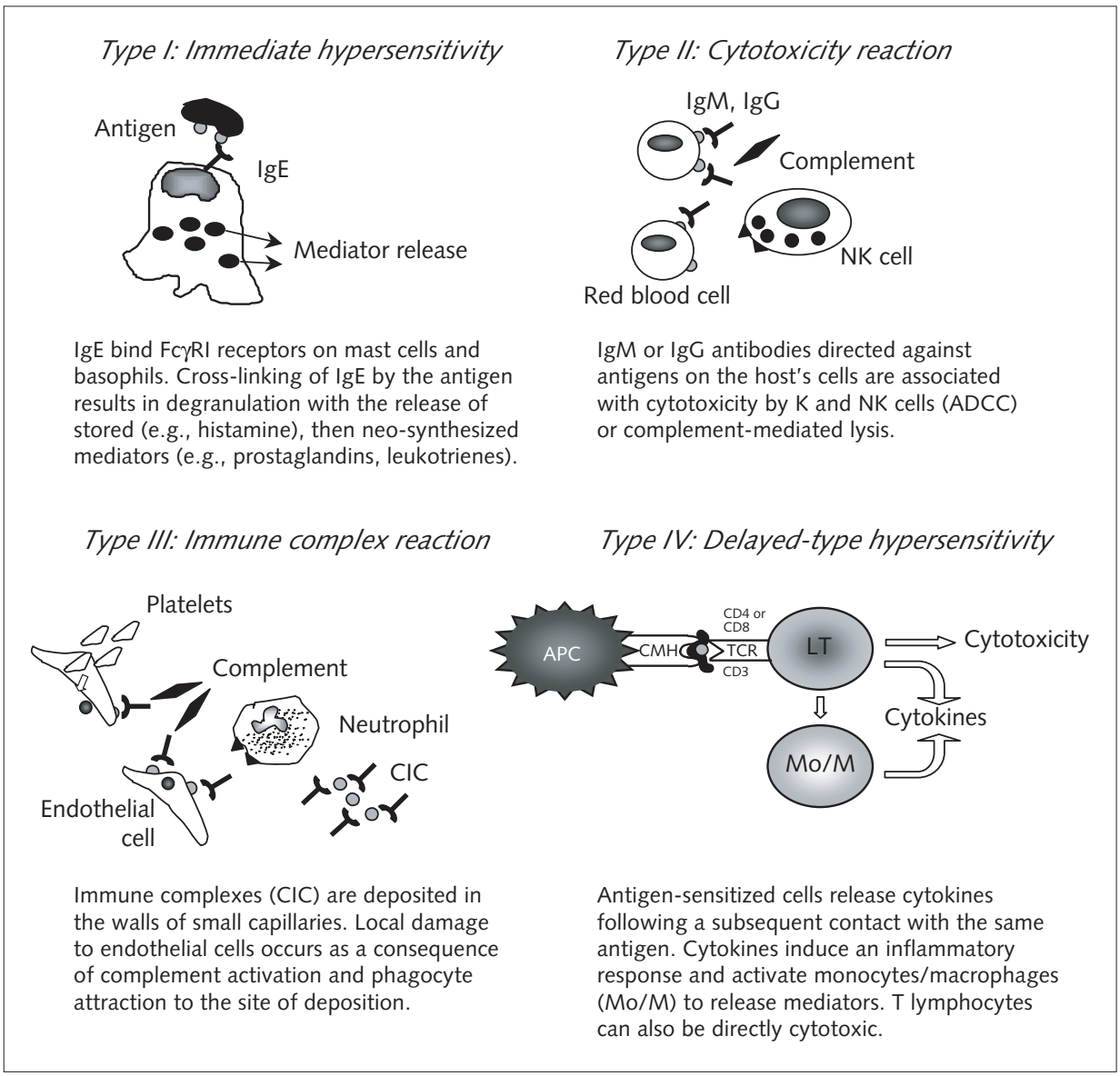

FIGURE 1
Mechanisms of drug-induced hypersensitivity reactions according to the Gell and Coombs classification.

granules. Another consequence of DEGRANULATION is *de novo* synthesis of mediators from membrane phospholipids including PROSTAGLANDINS and LEUKOTRIENES, whose release is therefore delayed. The clinical manifestations (ANAPHYLAXIS) are the direct consequences of the biological effects of released mediators (see chapter A7).

CYTOTOXICITY (type II) reactions are due to IgM or less often IgG ANTIBODIES (see chapter A3). Typically, when the sensitizing drug bound to the surface

of blood cells encounters circulating ANTIBODIES, the resulting complement activation provokes the destruction of blood cells as is seen in IMMUNOALLERGIC hemolytic anemias and thrombocytopenias.

Circulating immune complexes formed when the antigen is in greater quantity in the serum than IgM or IgG ANTIBODIES are the cause of classical type III reactions. Depending on their size, immune complexes deposit in capillary vessels and activate the COMPLEMENT SYSTEM, platelets, MACROPHAGES and NEUTROPHILS (see chapter A6). Activated cells release a variety of mediators and free radicals, which damage the endothelial cells. If the antigen is present predominantly at one site, localized damage is seen as in the ARTHUS REACTION. Immune complex deposition is one possible mechanism of cutaneous vasculitis. When the immune complexes are present in the circulation, they may cause serum sickness with fever, arthralgias, cutaneous eruption and proteinuria within 9–11 days after the injection of heterologous serum or MONOCLONAL ANTIBODIES. No immune complexes circulating in the blood or deposited in the glomeruli are seen after treatment with low-molecular-weight drugs. Therefore, the term serum sickness-like disease should be used to avoid confusion.

Delayed HYPERSENSITIVITY (type IV) reactions include allergic contact dermatitis and photoallergy. Contact dermatitis can be either non-immune-mediated (i.e., irritant contact dermatitis) or immune-mediated (i.e., allergic contact dermatitis). Allergic contact dermatitis is characterized by the infiltration of T LYMPHOCYTES into the dermis and epidermis. After penetrating into the skin, drugs or their metabolites can play the role of HAPTENS that bind to, or complex with various cells, including Langerhans cells and keratinocytes. Langerhans cells process and present the drug antigen to T LYMPHOCYTES (see chapter A2), which leads to the clonal proliferation of sensitized LYMPHOCYTES and to a clinically patent inflammatory reaction. Delayed HYPERSENSITIVITY reactions have been proposed to be subdivided into four distinct sub-categories [34]: type IV-a reactions involve a TH1 RESPONSE and closely correspond to the Gell and Coombs type IV reactions. Type IV-b reactions involve a TH2 RESPONSE in which the cytokine IL-5 is suspected to play a key role as in DRESS and drug-induced exanthemas. Type IV-c reactions are caused by CYTOTOXIC T LYMPHOCYTES and finally type IV-d reactions involving IL-8 lead to neutrophilic INFLAMMATION. This extended classification offers the advantage of sticking more closely to the wide spectrum of drug-induced HYPERSENSITIVITY reactions, but it remains to be established whether it is fully applicable to non-cutaneous drug-induced reactions involving T LYMPHOCYTE-mediated mechanisms.

Pseudo-allergic reactions

An immune-mediated mechanism does not account for all drug-induced HYPERSENSITIVITY reactions [35]. Indeed, clinical manifestations mimicking a genuine IgE-mediated reaction have been consistently described in some patients exposed for the first time to the same offending drug, hence the proposed term of PSEUDO-ALLERGIC or non-immune-mediated HYPERSENSITIVITY reactions. Several mechanisms have been identified.

HISTAMINE can be released by MAST CELLS and BASOPHILS by an IgE-independent (direct) mechanism (see chapter A7). A CYTOTOXIC or osmotic effect may be involved. Clinical signs mimic more or less closely a histaminic reaction with flush, redness of the skin, headache, cough and abdominal pain. The red man syndrome induced by vancomycin is one typical example [36]. Complement activation can be caused by immunological as well as non-immunological triggers, such as the pharmaceutical solvent Cremophor EL©, hydrosoluble radiological contrast media, and liposomes [37]. The ANAPHYLATOXINS, C3a and C5a, released during complement activation induce leukocyte CHEMOTAXIS, increased vascular permeability, contraction of bronchial smooth muscle, HISTAMINE release, LEUKOTRIENE generation, and IL-1 release. Direct complement activation has been advocated as one mechanism of infusion reactions associated with the therapeutic MONOCLONAL ANTIBODIES rituximab [38]. Aspirin as well as most NSAIDs can cause acute intolerance reactions that develop within 1 hour after ingestion often as an acute ASTHMA attack, possibly associated with rhinorrhea and conjunctival irritation [39]. Aspirin and the majority of NSAIDs inhibit the COX-1 isoform of the enzyme CYCLOOXYGENASE (COX) more potently than the COX-2

isoform (see chapters A7 and C14). Any NSAID with marked COX-1 inhibiting activity can precipitate ASTHMA attacks in patients with a genetic predisposition, resulting in hyperproduction of sulfidoleukotrienes (LTC4). That COX-2 inhibitors appeared to be safe in patients with a history of aspirin intolerance supports this hypothesis. Finally, angioedema associated with angiotensin-converting enzyme inhibitors probably results from the decreased degradation of BRADYKININ, which increases vascular permeability, contracts smooth muscles and elicits pain [40].

Autoimmunity

Although AUTOIMMUNITY largely remains a mystery, AUTOIMMUNE DISEASES are relatively common in the general population [41]. Estimates vary widely, but over 1 million new cases may develop every 5 years in the USA. Prevalence rates range from less than 5 per 100 000 to more than 500 per 100 000 depending on the AUTOIMMUNE DISEASE. Thus, although one given AUTOIMMUNE DISEASE can be considered to be rare, collectively AUTOIMMUNE DISEASES are rather frequent. A wealth of factors including drugs and chemical exposures [42, 43] are supposedly involved even though the underlying mechanisms are not understood.

It is noteworthy that in most instances one given drug can only induce one type of drug-induced autoimmune reaction, e.g. hydralazine and pseudolupus, or α-methyldopa and autoimmune hemolytic anemia. In contrast, immunostimulatory drugs, such as rIL-2 and interferon (IFN)-α, may be associated with a wide range of more frequent AUTOIMMUNE DISEASES that cannot be distinguished from spontaneous diseases [1].

Clinical manifestations of autoimmunity

AUTOIMMUNE DISEASES are clinically very diverse and in many instances the diagnosis is based on the presence of several clinical signs among a predefined set. The clinical presentation of drug-induced autoimmune reactions is more or less variable with respect to the spontaneous disease so that the presence of AUTOANTIBODIES in the sera of patients is a prerequisite. Spontaneous as well as drug-induced AUTOIMMUNE DISEASES are divided into systemic and organ specific.

Systemic autoimmune diseases

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) is estimated to affect 2–10 in 10 000 individuals. The causes of SLE are not known, but endocrine, genetic and environmental factors are likely to be involved. The lupus syndrome or pseudolupus, although it is the most common drug-induced autoimmune reaction, is a rare adverse event [44]. AUTOANTIBODIES have been detected in the sera of up to 25% of patients treated with hydralazine and 50% of those treated with procainamide. However, no clinical signs were associated with AUTOANTIBODIES in the majority of patients. Other drugs seldom reported to induce lupus syndromes include several anti-epileptic drugs, most β-blockers, chlorpromazine and isoniazid. In recent years, MINOCYCLINE-induced pseudolupus has been increasingly reported. Clinical and biological dissimilarities can be found between drug-induced lupus syndromes and SLE. In contrast to SLE, lupus syndromes are as frequent in men as in women. The most typical clinical signs of pseudolupus include arthritis (over 80% of patients), fever, weight loss, and muscular weakness with myalgias. Cutaneous manifestations are often uncharacteristic. Renal involvement is inconsistent and usually mild. Neurological signs are usually lacking. One major distinction is the high incidence of pleural effusion seen in up to 40% of pseudolupus patients, and pericardial effusion, which can result in cardiac tamponade. No biological abnormalities are characteristic of drug-induced lupus syndromes. Antinuclear ANTIBODIES are always present. Anti-double-stranded (ds) or native DNA ANTIBODIES are found in 50–70% of patients with SLE, but in less than 5% of those with the lupus syndrome. In contrast, ANTIBODIES to denatured DNA are relatively common in the lupus syndrome. No AUTOANTIBODIES have so far been identified as markers of drug-induced lupus syndromes. In contrast to SLE, lupus syndromes have a favorable outcome after cessation of the offending drug.

Scleroderma (or systemic sclerosis) is a relatively rare disease characterized by a more or less diffuse infiltration of the dermis and viscera by collagen with vascular abnormalities including vasospasm and microvascular occlusion. The pathogenesis of scleroderma has not been elucidated. There is an overproduction of collagen by fibroblasts. T LYMPHOCYTES are thought to play a pivotal role. Extremely few drugs have been reported to induce scleroderma-like diseases. The most severe was the oculo-mucocutaneous syndrome induced by the β-blocker practolol.

Organ-specific autoimmune diseases

In contrast to systemic autoimmune reactions induced by drugs, organ-specific reactions are often characterized by a HOMOGENEOUS ANTIBODY response against a unique TARGET and clinical symptoms closely mimicking those of the spontaneous AUTOIMMUNE DISEASE.

Guillain-Barré syndrome usually presents with progressive lower extremity weakness potentially leading to autonomic dysfunction. The mortality rate is 3–5%. A possible link with vaccination has been suspected, but the evidence is scarce [45]. The suspected role of hepatitis B vaccine in MULTIPLE SCLEROSIS, a multifocal demyelinating disease of the CENTRAL NERVOUS SYSTEM, once a matter of strong debate, has not been substantiated by the results of epidemiological studies [46]. Myasthenia is characterized by a loss of muscular strength due to impaired neuromuscular transmission [47]. There is a predilection for certain cranial nerves and virtually all patients complain of ocular symptoms. Of patients with the generalized disease, 80–90% have IgG AUTOANTIBODIES against the nicotinic RECEPTORS of ACETYLCHOLINE in the neuromuscular motor plates. PENICILLAMINE is the most frequent cause of drug-induced myasthenia. Whatever the causative drug, the underlying mechanism is not known.

Autoimmune thyroiditis typically presents as a slowly progressing atrophy of the thyroid gland due to a specific autoimmune response involving T cells and AUTOANTIBODIES [48]. Rarely, drugs, such as rIL-2 and the IFNs have been suspected to be responsible.

Mechanisms of drug-induced autoimmunity

Our understanding of the mechanisms involved in drug-induced AUTOIMMUNITY is extremely poor and largely limited to hypotheses and assumptions [49]. It is unknown to what extent, if any, findings in animal models of AUTOIMMUNE DISEASES or human patients apply to drug-induced autoimmune reactions. Therefore, the only few (sufficiently) conclusive data are presented.

Anti-thyroid AUTOANTIBODIES have been described in up to 30% of patients treated with rIL-2 and one likely explanation is that thyroid cells under the influence of IL-2-induced production of IFN-γ express MHC class II molecules and act as antigen-presenting cells with the production of antithyroid AUTOANTIBODIES as a consequence. [50]. Drug metabolites formed in the liver can bind to CYP450 isoforms, such as CYP1A2 (dihydralazine), CYP4E1 (halothane) or CYP2C9 (tienilic acid). Drug-induced (autoimmune or immunotoxic) hepatitis is the resulting consequence; extremely few drugs have so far been shown to be involved [51].

Molecular mimicry is another possible mechanism where part of a given protein closely resembles a part of another protein. Therefore, when a foreign protein enters the body, the IMMUNE SYSTEM mounts a specific ANTIBODY response, and when the foreign protein closely resembles a self-protein of the body, AUTOANTIBODIES are formed that can be pathogenic. The involvement of molecular mimicry in drug-induced AUTOIMMUNITY is only assumptive. T LYMPHOCYTES are a major focus of research on AUTOIMMUNITY. Recognition of closely similar EPITOPES shared by self and non-self molecules can trigger autoimmune responses due to molecular mimicry, or more subtle mechanisms. Activation of T cells could also be due to drugs mimicking COSTIMULATORY MOLECULES or MHC class II ANTIGENS that are exquisitely involved in the functioning of the immunological synapse.

Summary

Drug-induced HYPERSENSITIVITY and autoimmune reactions are potentially severe adverse events. The

clinical features are relatively well known, but much remains to be done to obtain a clear understanding of the underlying mechanisms. Because HYPERSENSITIVITY reactions are more frequent and the mechanisms better understood, a number of pending issues could be actively addressed, including better diagnosis tools and procedures to ensure that drug-related HYPERSENSITIVITY reactions are indeed correctly identified; the actual involvement of T LYMPHOCYTES in these reactions; the role of pharmacogenetic and immunogenetic backgrounds; and finally, the development of new models and techniques to predict drug allergenicity at the preclinical stage [52].

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Important websites

http://dermnetnz.org – Website of the New Zealand Dermatological Society presenting a lot of information and photos on dermatological reactions including drug-induced reactions

http://www.worldallergy.org – Website of the World Allergy Association, an international organization whose members consist of 84 regional and national allergology and clinical immunology societies from around the world

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Cancer immunity

Jan W. Gratama, Cor H. J. Lamers and Reno Debets

Introduction: Cancer immunity from a historical perspective

Almost 100 years ago, Ehrlich and coworkers observed the presence of infiltrates of mononuclear cells around or inside tumor lesions [1]. This finding led them to propose that tumors could be recognized and inhibited by the 'magic bullets' of the IMMUNE SYSTEM. At the end of the 19th century, studies were initiated that aimed at actively immunizing cancer patients against their own cancerous tissue. During the subsequent decades, cancer patients were non-specifically immune-stimulated with relatively crude leukocyte extracts such as transfer factor, immune-RNA, BACTERIAL EXTRACTS such as bacillus Calmette-Guérain, Coley's toxin or LEVAMISOLE. These studies were initiated in spite of the fact that little was known about the various components of the IMMUNE SYSTEM that could react against cancer, and even less was known about the structures on cancer cells that can be recognized by the IMMUNE SYSTEM.

The discrimination between 'self' and 'non-self' by the IMMUNE SYSTEM – the 1960 Nobel Prize-winning concept of Burnet and Medawar – has been pivotal for modern tumor immunology. Subsequently, Thomas and Burnet developed the 'immune surveillance' theory. The core of this theory is that the IMMUNE SYSTEM protects the host from cancer by detecting and destroying newly formed cancer cells, recognized as non-self [2, 3]. This immune surveillance theory, including its critics and the various experiments supporting and disproving it, has strongly influenced tumor immunological research during the past 40 years.

Ever since, the existence of immunity against cancer has been abundantly demonstrated in animal (mostly murine) models and in man. Interest in the clinical application of IMMUNOTHERAPY to treat cancer has been rekindled in the last two decades by the revival of the immunosurveillance theory, the discovery and structural characterization of TUMOR-ASSOCIATED ANTIGENS (TAA), our progress in understanding the molecular pathways required for the induction and maintenance of immune responses, and methodological advances to generate specific immunological probes in the form of tumor-specific CYTOTOXIC T LYMPHOCYTES (CTL) and MONOCLONAL ANTIBODIES (mAb). Results obtained until now have revealed:

- the increased susceptibility of immunodeficient patients to cancer as compared to immunocompetent individuals, supporting the concept of immunosurveillance against cancer [4];
- the molecular characterization of a wide range of various types of TAA on cancer cells, as detailed below [5];
- the feasibility of using TAA as vaccines and of using dendritic cells (DC) to induce tumor rejection in tumor-bearing animals and patients [6, 7];
- the efficacy of interleukin (IL)-2 and adoptively transferred T lymphocytes, expanded from tumor-infiltrating lymphocytes (TIL), in some patients with metastatic cancer, in particular melanoma and renal cell carcinoma [8–11];
- the immunosuppressive capacities of tumor cells, i.e., to secrete immunosuppressive cytokines such as transforming growth factor (TGF)-β and IL-10, and to inhibit leukocytes with antitumor capacities [12, 13].
- the potential of adoptive cellular immunotherapy, in combination with cytoreductive therapy, to induce long-lasting remissions in patients with cancer [14, 15].

Here, we discuss the key elements involved in the generation of antitumor responses: the cellular and humoral components of the immune system and the

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target antigens on the tumor cells. On this basis, we review the various immunotherapeutic approaches of cancer and place envisaged future developments into the perspective of current experience.

Key players in the immune responses against cancer

Both innate and adaptive components of the IMMUNE SYSTEM interact to generate antigen-specific immune responses. As detailed in the previous chapters, the INNATE IMMUNE SYSTEM constitutes the body's first line of defense against 'foreign invaders'. INNATE IMMUNITY involves a large number of different cell populations such as epithelial cells, MONOCYTES, MACROPHAGES, DC, POLYMORPHONUCLEAR LEUKOCYTES or GRANULOCYTES, and some lymphocyte subsets that are at the interface between innate and ADAPTIVE IMMUNITY [NATURAL killer (NK) LYMPHOCYTES, cluster of differentiation (CD)5$^+$ B LYMPHOCYTES, T cell RECEPTOR (TCR)-γδ$^+$ T LYMPHOCYTES and NATURAL killer T (NKT) LYMPHOCYTES]. The INNATE IMMUNE SYSTEM also comprises a variety of humoral factors such as CYTOKINES, CHEMOKINES, enzymes (e.g., LYSOZYME), metal-binding proteins, integral membrane ion transporters, complex carbohydrates and complement. The phagocytic cells (MACROPHAGES, GRANULOCYTES) and the COMPLEMENT SYSTEM constitute effector mechanisms by which the 'invaders' can be destroyed. The production of CYTOKINES and CHEMOKINES acts in concert with antigen presentation by DC and MONOCYTES to initiate adaptive immune responses. Figure 1 shows how the killing of TARGET cells by NK LYMPHOCYTES is guided by the balance between activating and inhibitory signals.

ADAPTIVE IMMUNITY makes use of a unique mechanism whereby genetic mutations occurring in two specialized cell populations, B and T LYMPHOCYTES, produce numerous molecular 'shapes' that are expressed as ANTIBODIES (Ab) and TCR. Figure 2 provides a simplified overview of how the effector components of the adaptive IMMUNE SYSTEM (T cells and Ab) are regulated and eliminate their targets. Antigen-SPECIFIC IMMUNITY is generated when Ab and TCR are expressed and up-regulated through the formation and release of CYTOKINES and CHEMOKINES. Thus, ADAPTIVE IMMUNITY involves a wide range of antigen RECEPTORS expressed on the surface of T and B LYMPHOCYTES to detect 'foreign' molecules. B LYMPHOCYTES respond to antigen by secreting their own antigen RECEPTORS as Ab after having differentiated into plasma cells. Ab interact with effector mechanisms *via* binding of their constant part (Fc) to complement, Fc-RECEPTOR-bearing PHAGOCYTES and Fc-RECEPTOR-bearing CYTOTOXIC (NK and T) LYMPHOCYTES.

The major T LYMPHOCYTE SUBSETS are characterized by expression of the differentiation markers CD4 or CD8. While Ab mostly react with intact proteins or carbohydrates, T cells mostly react with peptides expressed on the cellular surface *via* presentation by scaffolds, i.e., molecules of the MAJOR HISTOCOMPATIBILITY COMPLEX (MHC), on antigen-presenting cells (APC). One of the most potent types of APC is the DC. The DC pick up ANTIGENS in the skin or other peripheral tissues, and migrate to the T cell zones of LYMPHOID ORGANS where they stimulate naive CD4$^+$ and CD8$^+$ T cells.

Most CD4$^+$ T cells are 'T helper (Th) cells' and recognize antigens in the form of 15- to 25-mer peptides presented by MHC class II molecules. These molecules present peptides that are mainly derived from the extracellular compartment, as opposed to peptides presented by MHC class I molecules that capture endogenously processed peptides (see below) [16]. CD4$^+$ T cells are important immunoregulatory cells. They recruit and activate other immune cells such as B cells, CD8$^+$ T cells, MACROPHAGES, MAST CELLS, NEUTROPHILS, EOSINOPHILS and BASOPHILS. CD4$^+$ Th cells are classified into four subsets, Th1, Th2, Th17 and regulatory T cells (Treg), based on their functions, their patterns of cytokine secretion and expression of specific transcription factors [17]. Th1 cells mediate immune responses against intracellular pathogens and play a particularly important role in resistance to mycobacterial infections. Th1 cells are important for CELL-MEDIATED IMMUNITY by PHAGOCYTES, and secrete "inflammatory" type-1 CYTOKINES such as interferon (IFN)-γ and IL-2. Th2 cells are associated with neutralizing ANTIBODY responses, mediate host defense against extracellular parasites, and produce type-2 CYTOKINES such as IL-4, IL-5 and IL-13, which are associated with allergic responses [18, 19]. The more recently described Th17 cells are generated by CYTO-

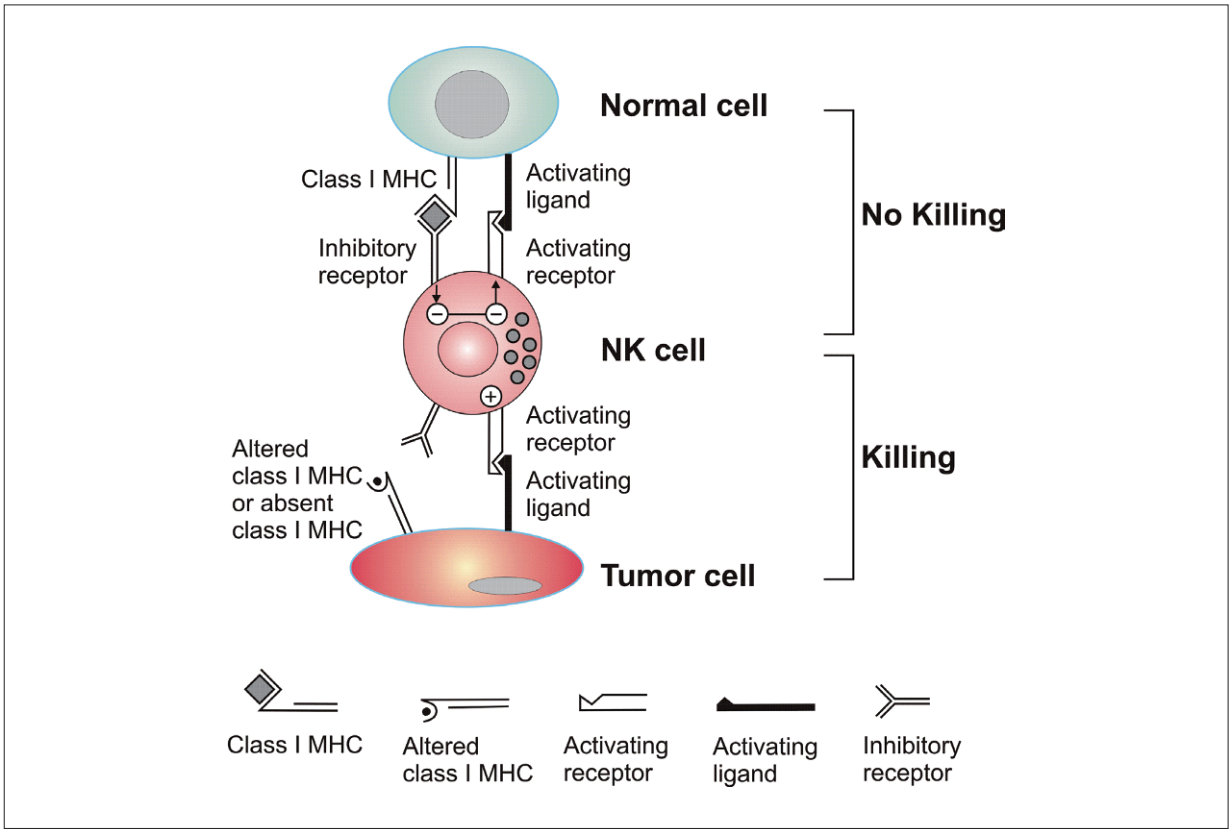

FIGURE 1. SURFACE RECEPTOR INTERACTIONS ESTABLISHING THE BASIS FOR TOLERANCE AND CYTOLYTIC RESPONSES BY NK CELLS

NK cell activation is regulated by a balance of signals derived from activating and inhibitory surface receptors. Activating receptors recognize an array of surface molecules, some of which are expressed ubiquitously on surfaces of other cells of the body. The so-called "killer-inhibitory receptors" (KIR) recognize MHC class I molecules, which are expressed by most normal cells, while many tumor cells lack expression of these "self" molecules. Interaction between KIR and MHC class I triggers an inhibitory signal within the NK cell that dominantly suppresses activating receptor ligands, and result in tolerance. Upon encounter with a tumor cell lacking MHC class I ("missing self"), the lack of inhibitory signaling triggers rapid and direct release of cytolytic granules by the NK cell, resulting in specific cytolysis of the tumor cell. Adapted from [90].

KINES released by activated DC, and mediate acute INFLAMMATION at sites of infection [20]. Finally, Treg cells can suppress both innate and adaptive immune responses, and thus maintain TOLERANCE and limit immunopathology. Treg cells are identified based on their high levels of surface CD25 (IL-2Rα) and expression of the transcriptional regulator forkhead box P3 (FoxP3) [21, 22]. As observed in all other adaptive immune responses, Treg cell activation is antigen specific, which implies that suppressive activities of Treg cells are antigen dependent. Importantly, the presence of tumor-specific $CD4^+$ T regulatory cells at tumor sites may inhibit T cell responses against cancer (see below) [23].

$CD8^+$ T LYMPHOCYTES all differentiate into CTL that kill their TARGET cells. As their actions are potentially

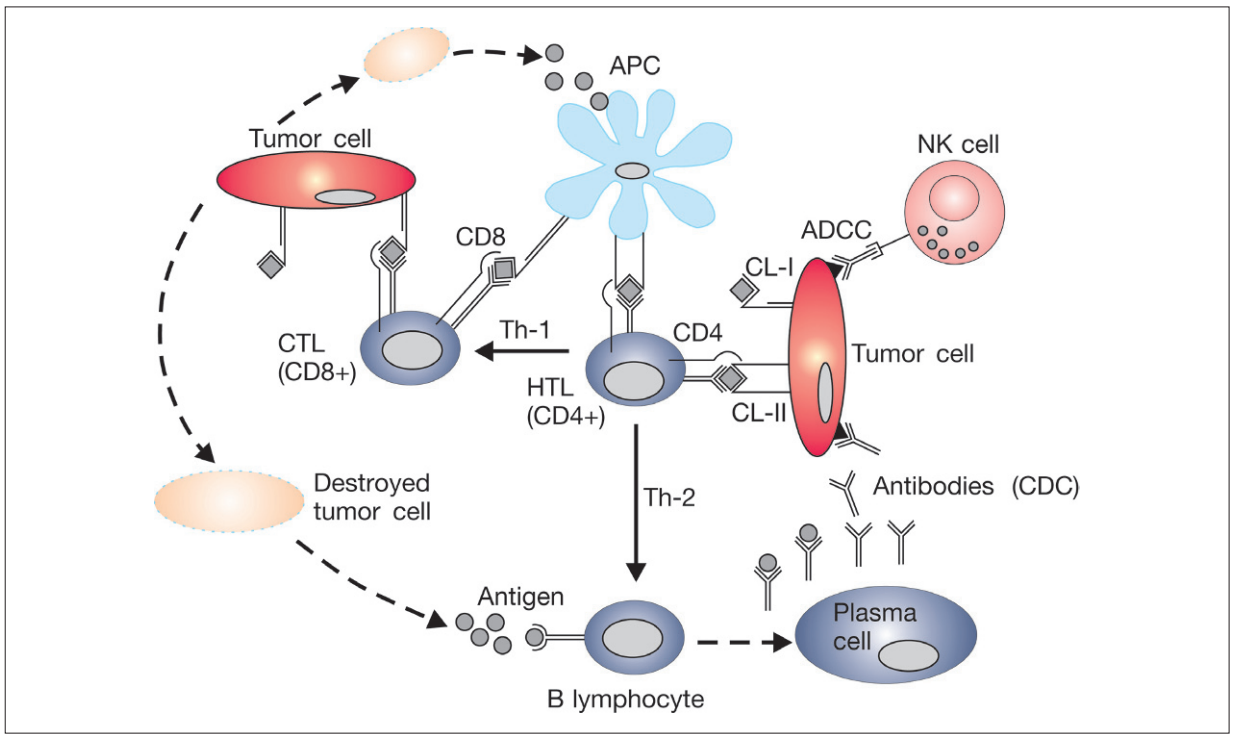

Figure 2. Key players of the immune system in mounting an antitumor response

A 'professional' antigen-presenting cell (APC) presents antigenic peptides (depicted as diamonds) to a helper T lymphocyte (HTL, Th) via its MHC class II molecules (CL-II), and to a cytotoxic T lymphocyte (CTL) via its MHC class I molecules (CL-I). The Th cells may also recognize antigen on a tumor cell itself if the tumor cells express MHC class II molecules (right side of figure); similarly, a CTL may also recognize antigen on a tumor cell itself if the tumor cells express MHC class I molecules (upper left). The Th cells recognize antigen through their T cell receptors for antigen (TCR), which are supported in this task by CD4 coreceptor molecules. By the same token, CTL recognize antigen by their TCR supported by CD8 co-receptor molecules. Other important accessory molecules and their receptors, as well as Th17 and regulatory T cells, are not shown here (see text for details). The Th cells support CTL activation and proliferation by secreting so-called Th1 cytokines, of which IL-2 and IFN-γ are the most important. Th2 cells stimulate B lymphocytes through specific cytokines (mainly IL-4 and IL-5). B cells recognize soluble antigen through their B cell receptor for antigen; upon activation, B cells differentiate into plasma cells, which secrete antibodies specific for that antigen, and which may exert their effector function through complement-dependent cytotoxicity, and in collaboration with NK cells through antibody-dependent cellular cytotoxicity (ADCC). Upon engagement of their TCR and accessory molecules, $CD8^+$ and also $CD4^+$ T cells can destroy tumor cells by secreting granzymes, perforins and cytokines such as TNF-α, or by up-regulation of CD95 (Fas ligand) on tumor cells. The remnants of destroyed tumor cells can be taken up by APC, processed and presented to T cells; in addition, they may be specifically recognized by antibodies and eliminated via Fc-receptor-expressing phagocytes.

destructive, they require costimulatory activity to become activated. This primarily comes from $CD4^+$ T cells in addition to DC. $CD8^+$ T cells react with eight- to ten-mer peptides presented on the cellular surface by the scaffolds formed by their MHC class I molecules. These peptides contain two to three so-called

'anchoring residues' that fit into specific pockets of the MHC class I molecule [24]. $CD8^+$ T cells destroy their TARGET cells, after attachment of (i) their TCR to the appropriate MHC-peptide complex, and (ii) their accessory molecules (such as CD8 and CD28) to the corresponding ligands, by perforating their membranes with enzymes (i.e., perforin, granzymes and granulysin) or by triggering a process of self-destruction (i.e., APOPTOSIS). In this way, $CD8^+$ T cells can move from one tumor cell to another expressing the same MHC-peptide complexes, kill them selectively, and thus can mount a very specific and robust antitumor response.

In the setting of ADOPTIVE IMMUNOTHERAPY of cancer (see below), the administration of autologous $CD4^+$ Th cells, concurrently with $CD8^+$ CTL, has been shown to prevent exhaustion of the infused $CD8^+$ CTL [25, 26]. $CD4^+$ T cells activate DC through cross-linking of CD40, which thus provide enhanced antigen presentation and COSTIMULATION, which leads to priming of $CD8^+$ CTL function [27]. Thus, $CD4^+$ T cell help results in *de novo* generation of tumor-specific $CD8^+$ CTL and concomitant tumor destruction [28]. The exact requirement for $CD4^+$ T cell help during priming may depend on the nature of the stimulus, but the requirement for $CD4^+$ T cells during memory responses is beyond dispute [29]. We propose that $CD4^+$ T cells play a critical role in anti-tumor immunity that goes beyond the mere induction and maintenance of tumor-specific $CD8^+$ CTL. Specifically, $CD4^+$ T cells activate $CD8^+$ CTL already present at the site of the tumor and enable other $CD8^+$ CTL to migrate into the tumor, induce an anti-tumor Ab response, and otherwise contribute to tumor regression through production of IFN-γ, which inhibits tumor-induced angiogenesis [25, 30].

CYTOKINES produced by $CD4^+$ Th cells also regulate MACROPHAGES by stopping their migration after their engagement, allowing them to accumulate at a particular site. Thus, more efficient PHAGOCYTOSIS is stimulated, so that increasing numbers of 'invaders' are efficiently destroyed. $CD4^+$ T cells amplify themselves by secreting CYTOKINES and interacting with them; IL-2 is a key example of this 'autocrine loop.' This action enhances the Th cell response and thus the entire IMMUNE SYSTEM's response to foreign ANTIGENS.

Expression of targets for the immune system by cancer cells

Tumor-associated antigens recognized by T lymphocytes

Clinical immunotherapeutic studies of cancer performed using *in vitro* expanded T LYMPHOCYTES isolated from tumors (see below) have demonstrated that $CD8^+$ T cells can specifically lyse, in an MHC-restricted fashion, autologous tumor cells or tumors arising from different tissues, but not normal cells [31, 32]. MHC class I molecules are expressed on the surface of most nucleated cells. To identify these MHC-restricted tumor ANTIGENS, cDNA or DNA COSMID pools derived from tumor cells have (transiently) been transfected into cell lines expressing the relevant MHC class I molecules. These transfected cell lines were then assayed for their ability to specifically stimulate cytokine production by MHC class I-matched, tumor-reactive T cell CLONES. The subsequent transfection of individual cDNAs or cosmids from pools that specifically stimulated the T cell CLONES allowed the identification of individual TAA. The first human TAA that was identified in this way by screening a large genomic library from an human leukocyte antigen (HLA)-A^*0101^+ melanoma patient was termed melanoma antigen (MAGE)-1 [33]. To identify the MAGE-1 peptide presented by HLA-A*0101, selected 9-mer peptides were synthesized and screened for their capacity to specifically stimulate the MAGE-1-specific CYTOTOXIC T cell CLONE isolated from this patient [34]. Since then, this 'reverse immunology' approach has been used to identify a large number of TAA (see below and Tab. 1). The definition of MHC class I peptide-binding motifs, i.e., the nature and position of pockets along the peptide binding groove of the MHC molecule that bind the anchoring residues of the peptides, has allowed the computer-assisted screening of large numbers of potentially immunogenic peptides including many TAA [24].

The manufacturing and use of MHC class II-binding peptides has thus far met with more problems than that of MHC class I-binding peptides due to: (i) the significantly weaker AFFINITY of interactions between MHC class II and the TCR; and (ii) the fact

Table 1. Overview of tumor-associated antigens studied in the National Cancer Institute pilot project for the acceleration of translational research [5].

Antigen	Description	Cum. score	Therap. function	Immuno-genicity	Ref.
I. Absolute specificity (*n*=10)					
LMP2	Latent membrane protein-2	0.78	0.75	1.0	[91]
HPV E6 E7	Human papilloma virus E6 & E7 protein	0.77	0.89	1.0	[42, 92]
EGFRvIII	Epidermal growth factor receptor type variant III	0.77	0.76	1.0	[93]
Idiotype	Idiotype expressed by B cell lymphoma	0.75	0.76	1.0	[94]
ras mutant	Mutation of k-ras oncogene	0.60	0.10	1.0	[95]
p53 mutant	Mutation of protein-53 (unique random mutations)	0.58	0.35	1.0	[96]
bcr-abl	Oncogene fusion protein consisting of BCR and ABL	0.56	0	1.0	[97]
Sarcoma translocation breakpoints	Occurring in Ewing's sarcoma family of tumors or alveolar rhabdomyosarcoma	0.54	0	0.39	[98]
ERG	Fusion product between transmembrane protease, serine 2 and ERG transcription factor	0.48	0	0.39	[99]
ETV6-AML	Fusion product of ETV6 and AML1 genes	0.37		0	[100]
II. Oncofetal antigens (*n*=16)					
WT-1	Wilms tumor protein	0.81	0.75	1.0	[101]
MAGE A3	Melanoma-associated antigen 3	0.71	0.79	1.0	[102]
NY-ESO-1	New York-esophagus antigen 1	0.66	0.75	1.0	[103]
AFP	Alpha-feto protein	0.49	0.15	1.0	[104]
PAX3	Paired box (PAX) family of transcription factors	0.47	0	1.0	[105]
MYCN	myc myelocytomatosis viral related oncogene	0.42	0	0.39	[96]
MAGE A1	Melanoma-associated antigen 1	0.40	0	1.0	[106]
mBORIS	Artificially mutated brother of regulator of imprinted sites (BORIS) transcription factor	0.38	0.10	0.11	[107]
PLAC1	Gene encoding putative placental cell surface protein	0.39	0	0.39	[108]
OY-TES-1	Proacrosin binding protein sp32 precursor	0.32	0	0.10	[109]
SP17	Sperm protein 17	0.30	0	0.11	[110]
AKAP-4	A-kinase anchor protein 4	0.26	0.10	0.11	[111]
SSX-2	Synovial sarcoma, X breakpoint 2	0.26	0	0.39	[112]
XAGE-1	Splice variant of X-chromosome-encoded XAGE-1 antigen	0.23	0	0.10	[113]
MAD-CT-1	Melanoma cancer testis antigen-1	0.15	0	0.10	[114]
MAD-CT-2	Melanoma cancer testis antigen-2	0.14	0	0.10	[115]

Table 1 (continued)

Antigen	Description	Cum. score	Therap. function	Immuno-genicity	Ref.
III. Overexpressed antigens					
Her2/neu	Human epidermal growth factor receptor 2	0.75	0.85	1.0	[116]
p53 non-mutant	Non-mutated p53	0.67	0.42	1.0	[117]
GD2	Disialoganglioside	0.65	0.75	1.0	[118]
CEA	Carcinoembryonic antigen	0.62	0.75	1.0	[119]
proteinase 3	Serine protease expressed in granulocytes	0.57	0.70	1.0	[120]
survivin	Inhibitor of apoptosis	0.55	0.10	1.0	[121]
hTERT	Human telomerase reverse transcriptase	0.54	0.15	1.0	[122]
EphA2	Ephrin type A receptor 2	0.53	0.10	1.0	[123]
ML-IAP	Melanoma inhibitor of apoptosis protein	0.50	0	1.0	[124]
EpCAM	Epidermal cell adhesion molecule	0.48	0.10	1.0	[125]
NA17	N-Acetyl glucosaminyl-transferase V	0.48	0.59	1.0	[126]
ALK	Fusion product between anaplastic lymphoma kinase (ALK) and nucleoplasmin gene	0.46	0	0.39	[127]
Androgen receptor	Overexpressed in prostate cancer	0.45	0.10	0.39	[128]
Cyclin B1	Cell cycle regulatory protein involved in G2/ mitosis	0.44	0.10	0.39	[129]
RhoC	GTPase, member of Ras gene family	0.42	0	0.39	[130]
GD3	Ganglioside GD3	0.41	0	1.0	[131]
fucosyl GM1	Fucosyl-GM1 ganglioside	0.41	0	1.0	[132]
mesothelin	Overexpressed in mesothelioma and pancreatic cancer	0.41	0	1.0	[133]
CYP1B1	Carcinogen activator cytochrome P450 1B1	0.40	0	1.0	[134]
sLe(a)	Sialyl Lewis(a) adhesion molecule	0.40	0	1.0	[135]
GM3	Ganglioside GD3	0.38	0.10	1.0	[136]
Globo H	Hexasaccharide portion of globo H glyco-ceramide	0.37	0	1.0	[137]
RGS5	Regulator of G protein signaling 5	0.35	0	1.0	[138]
SART3	Squamous cell carcinoma antigen recognized by T cells	0.35	0	1.0	[139]
carbonic anhydrase IX	Transmembrane protein overexpressed in renal cell cancer	0.34	0	1.0	[140]
LCK	Leukocyte-specific protein tyrosine kinase	0.28	0	1.0	[141]
HMWMAA	High molecular weight melanoma-associated antigen	0.27	0.10	0.11	[142]
B7H3	Member of B7 family of T cell costimulatory proteins	0.22	0	0	[143]
Legumain	Asparaginyl endopeptidase overexpressed by tumor-ass. m'phages	0.19	0.10	0.11	[144]

Table 1 (continued)

Antigen	Description	Cum. score	Therap. function	Immuno-genicity	Ref.
IV. Tissue-specific differentiation antigens (*n*=12)					
PSMA	Prostate-specific membrane antigen	0.65	0.75	1.0	[145]
Melan-A/MART-1	Melanoma antigen recognized by T cells	0.60	0.77	1.0	[146]
gp100	Glycoprotein-100	0.59	0.75	1.0	[147]
tyrosinase	Enzyme catalyzing the production of melanin	0.56	0.65	1.0	[148]
PSA	Prostate-specific antigen	0.55	0.75	1.0	[149]
PAP	Prostatic acid phosphatase	0.52	0.69	1.0	[150]
polysialic acid	Side chain of NCAM, largely restricted to small-cell lung cancer	0.44	0	1.0	[151]
TRP-2	Tyrosinase-related protein 2	0.42	0.10	1.0	[152]
PSCA	Prostate stem cell antigen	0.41	0.75	0.11	[153]
NY-BR-1	Mammary gland differentiation antigen	0.36	0	0.39	[154]
PAX5	Regulator of G protein signaling 5	0.33	0	0.39	[155]
PAGE4	Prostate-associated antigen family member 4	0.17	0	0	[156]
V. Post-translational (*n*=4)					
MUC1	Mucin 1	0.79	0.75	1.0	[157]
Tn	7-Valent carbohydrate vaccine (GM2, Globo-H, Lewis Y, sialyl Tn, Tn, Thompson-Friedreich Ag, mucin 1)	0.37	0	1.0	[158]
STn	Sialyl Tn	0.34	0	1.0	[159]
Tie2	Angiopoietin-binding cell surface receptor 2	0.18	0.10	0.11	[160]
VI. Stromal (*n*=4)					
VEGFR2	Vascular endothelial growth factor receptor type 2 (stromal)	0.16	0.10	0.11	[161]
FAP	Fibroblast activation protein (stromal)	0.14	0.10	0	[162]
PDGFR-beta	Platelet-derived growth factor receptor-beta	0.14	0	0.11	[163]
Fra-1	Fos-related antigen (transcription factor)	0.13	0.10	0.11	[164]

that the CD4 coreceptor does not stabilize the interaction between class II multimer and TCR, as CD8 does for MHC class I multimers [35].

Antigens recognized by antibodies

In an individual, both cellular and humoral immune responses can be found that are independent of each other and also directed against different EPITOPES. ANTIGENS recognized by Ab are mostly intact proteins or carbohydrates, and are not presented by MHC molecules. Therefore, such ANTIGENS cannot be recognized by T LYMPHOCYTES *via* their TCR, which only recognize MHC-presented peptides. Many of these TAA are merely overexpressed by tumor cells in comparison to their normal counterparts. Although these TAA are not necessarily 'tumor-specific' in

the strict immunological sense, they can be used effectively to selectively TARGET the IMMUNE SYSTEM to tumors, resulting in clinical antitumor effects [36]. Using mAb, tissue-specific ANTIGENS have been identified that, although not tumor-specific, can serve as TARGET structures for immunotherapeutic interventions (see below).

A systematic review of 145 studies on humoral immune responses to TAA was primarily based on immunoassays [main techniques, enzyme-linked immunosorbent assay (ELISA) (77%) and Western blotting (20%)] [37]. Most of the 107 different TAA studied were derived from overexpressed or mutated proteins with expression patterns that were cytoplasmic in 42%, predominantly nuclear in 26%, membrane-bound in 21%, and extracellular (i.e., extracellular matrix or secreted) in 10%. Only a minority of the 107 ANTIGENS was analyzed in multiple studies; those that were addressed in 5 or more studies are shown in Table 2.

TAA-specific ANTIBODIES are only found in 0–3% of healthy individuals and seem to be highly specific for cancer, but average ANTIBODY frequencies against single ANTIGENS in patients with tumors (not selected for TAA expression) rarely exceed 15% [37]. Thus, a diagnostic assay will not be based on the presence or absence of ANTIBODIES against TAA. An exception to the pattern that healthy controls do not have TAA-specific ANTIBODIES is formed by the MUC1 core peptide for the secretory mucin protein; IgM ANTIBODIES against this peptide were found in 53% and IgG ANTIBODIES in 23% of individuals. This result may be explained by hypoglycosylation of immunogenic MUC1 EPITOPES that may occur in benign conditions (e.g., INFLAMMATION).

Overexpression of TAA may be reflected in the frequencies of humoral responses against these TAA. Most data exist for protein-53 (p53), where overexpression or mutations leading to extensive intracellular p53 accumulation may be associated with a high prevalence of anti-p53 ANTIBODIES. For New York-esophagus (NY-ESO)-1, ANTIBODY frequency was higher (up to 83%) in patients with tumors expressing this TAA, whereas such ANTIBODIES were not observed in patients with NY-ESO-1-negative tumors. Likewise, 82% of breast cancer patients with strong expression of human epidermal recertor-2/neurological (Her2/neu) have TAA-specific ANTIBODIES compared to patients with weak Her2/neu expression who have no such ANTIBODIES. ANTIBODIES against several TAA, such as p53, NY-ESO-1, survivin and tyrosinase,

TABLE 2. TUMOR-ASSOCIATED ANTIGENS (TAA) ELICITING HUMORAL IMMUNE RESPONSES AS STUDIED IN AT LEAST 5 STUDIES PER TAA [37]

TAA	Studies (*n*)	Tumor sites (*n*)	Tested sample (*n*)
p53	37	17	7764
MUC1 core peptide	13	7	2136
NY-ESO-1	13	11	1528
c-myc	9	7	2968
Survivin	9	8	2132
p62	6	11	2773
cyclin B1	6	6	2353
Her2/neu	5	3	771

p53, protein 53; MUC1, mucin 1; NY-ESO-1, New York-esophagus antigen 1, c-myc, transcription factor; survivin, inhibitor of apoptosis; p62, signaling adaptor involved in the activation of the transcription factor NF-κB; cyclin B1, cell-cycle regulatory protein involved in G2 → mitosis; Her2/neu, human epidermal growth factor receptor 2.

were found more frequently in advanced tumor stages, indicating the importance of antigen load and duration of antigen exposure for the induction of humoral TAA responses.

Humoral immune responses against p53 have been shown to be associated with poor prognosis. While such responses may be primarily related to increased exposure to the antigen, p53-specific ANTIBODIES may also reflect mutations of the protein that lead to more aggressive tumor behavior. In contrast, associations of ANTIBODIES with favorable prognosis were repeatedly reported for MUC1 ANTIBODIES and may be related to interference of ANTIBODIES with MUC1-induced tumor growth-promoting mechanisms. A similar cancer-suppressive mechanism has been suggested for endogenous ANTIBODIES directed against Her2/neu [37].

An important alternative strategy to study humoral immune responses to cancer is the serological identification of ANTIGENS by RECOMBINANT expression cloning (SEREX). This technique uses diluted sera from patients with cancer to screen tumor cDNA libraries expressed in *Escherichia coli* to identify ANTIGENS that have elicited high-titer IgG Ab [38]. The first application of SEREX revealed the NY-ESO-1 antigen using serum from a patient with squamous cell carcinoma of that organ [39]. Subsequently, a multitude of ANTIGENS from tumors of many histologies, including melanoma and colon, lung and renal cancers have been discovered using SEREX. The function of many of the newly identified proteins is still unknown. Importantly, screening for high-titer IgG TAA-specific Ab should identify patients with $CD4^+$ T cell responses to the TAA in question, since such a response is required for Ig CLASS SWITCHING. Indeed, MHC class II-restricted TAA-specific $CD4^+$ T cells, as well as MHC class I-restricted $CD8^+$ T cell responses to the same tumor-derived proteins as those identified by the high-titer IgG TAA-specific Ab have been detected in some of these cases [40, 41].

Classification of tumor-associated antigens

The task of ranking cancer ANTIGENS is enormous, and the number of potential cancer ANTIGENS is almost limitless. In 2009, the National Cancer Institute published a priority-ranked list of cancer vaccine TARGET ANTIGENS based on pre-defined and pre-weighted objective criteria established by a panel of experts [5]. This prioritization of TAA allowed the development of a list of "ideal" cancer antigen characteristics. Relative weights were assigned to those criteria, for which 75 representative ANTIGENS (listed in descending order of their overall weighting in Tab. 1) were selected for comparison and ranking. Information was assembled on the pre-defined criteria for the selected ANTIGENS, and the ANTIGENS were ranked on the basis of the pre-defined, pre-weighted criteria. Using this approach, the result of criteria weighting, in descending order, was as follows: (1) therapeutic function (32% of overall weight); (2) immunogenicity (17%); (3) role of the antigen in oncogenicity (15%); (4) SPECIFICITY (15%); (5) expression level and percent of antigen-positive cells; (6) cancer stem cell expression (7%); (7) number of patients with antigen-positive cancers (4%); (8) number of antigenic EPITOPES (4%); and (9) cellular localization of antigen expression (2%).

From a cancer immunologist's viewpoint, SPECIFICITY of antigen expression is an important tool to categorize the many ANTIGENS. We approached this issue by discussing the 75 ANTIGENS mentioned above as a function of their SPECIFICITY (Tab. 1). We divided the 75 ANTIGENS into 6 subgroups on the basis of their SPECIFICITY profiles as discussed below.

Ten ANTIGENS were classified as 'absolutely specific' (subgroup I). Two ANTIGENS with the highest cumulative scores as candidate vaccine ANTIGENS were 'virus-encoded proteins', which have been identified as sources of TAA in tumors that emerge from virally transformed cells. These are the latent membrane protein (LMP)-1 of the Epstein-Barr virus (EBV) that is expressed by nasopharyngeal carcinoma and EBV^+ Hodgkin's lymphoma, and the human papilloma virus (HPV)-encoded E6 and E7 proteins expressed by $HPV\text{-}16^+$ cervical carcinoma. These proteins typically contribute to malignant transformation. As these ANTIGENS are not expressed by normal cells, their usefulness as targets for IMMUNOTHERAPY of cancer appears to be straightforward. Recently, induction of HPV-16-SPECIFIC IMMUNITY has been shown to induce complete antitumor responses in patients with vulvar intra-epithelial neoplasia [42].

Second, truly tumor-specific ANTIGENS can arise from the fusion of distant genes resulting from translocation of chromosomes in tumor cells (bcr-abl, sarcoma translocation breakpoints, ERG and ETV6-AML). The candidate ANTIGENS are derived from the region immediately surrounding and containing the fusion site. Although these ANTIGENS are highly specific targets for IMMUNOTHERAPY, their use for this purpose is limited, as only a few EPITOPES have been observed to bind efficiently to a small subset of class I molecules and be naturally processed from the protein [43].

Third, unique tumor ANTIGENS can also arise from point mutations of normal genes whose molecular changes often accompany neoplastic transformation or tumor progression. These mutational ANTIGENS are only expressed by tumor cells and not by their normal counterparts. In addition to their limited immunogenicity for T cell responses (similar to the fusion proteins mentioned above), their therapeutic utility is limited because induced immune responses are restricted to those individual tumors expressing these ANTIGENS. These include mutations of EGRFRvIII and ras and the mutated forms of the p53 tumor suppressor protein, including those having unique random mutations of the p53 tumor suppressor protein specific for each patient. By the same token, the unique immunoglobulin IDIOTYPE expressed by each B cell lymphoma may constitute a potential TARGET for IMMUNOTHERAPY.

Sixteen ANTIGENS were classified as "oncofetal", i.e., had fetal expression with no or little expression in adult tissue (subgroup II). This group includes the "cancer-testis" ANTIGENS, so-called for their expression by histologically different tumors and, among normal tissues, by spermatogonia and spermatocytes of the testis and occasionally by placental trophoblasts (i.e., MAGE A3 and A1, MAD-CT1 and MAD-CT2). The expression of oncofetal ANTIGENS by tumors results from reactivation of genes that are normally silent in adult tissues but are transcriptionally activated in some tumors. Oncofetal ANTIGENS are less immunogenic than differentiation ANTIGENS (see below), and do not elicit 'spontaneous' CTL responses.

The largest group of TAA ($n=29$) comprises ANTIGENS that are 'overexpressed' by tumors arising from a variety of tissues, but also (albeit at much lower levels) by normal tissues (subgroup III). This high level of expression by tumor cells as revealed by MICROARRAY or DIFFERENTIAL DISPLAY ANALYSIS has been instrumental for the identification of many of these ANTIGENS. Several mAb have been raised against TAA from this group, and are currently used for cancer IMMUNOTHERAPY (see below).

A further 12 ANTIGENS have tissue-specific expression in normal adult tissue but are not essential for the individual's survival (e.g., prostate tissue and melanocytes; subgroup IV). These ANTIGENS are expressed by normal and neoplastic cells of the same lineage. These ANTIGENS can elicit 'spontaneous' $CD8^+$ CTL responses in cancer patients and healthy individuals. In melanoma, increased frequencies of these CTL are seen, but these are functionally inactive (i.e., anergic) [44]. A point of concern with the use of these ANTIGENS as targets for cancer IMMUNOTHERAPY is that normal cells expressing these ANTIGENS will also be destroyed, as illustrated in a clinical trial of melanoma patients treated with *ex vivo*-expanded TIL specific for MART-1 and gp100 [8].

For 4 further ANTIGENS, the core protein is expressed in normal tissue, whereas cancer cells have unique post-translational changes (such as glycosylation or phosphoryation; subgroup V). For the 4 remaining ANTIGENS, the normal antigen was expressed on tumor stroma ($n=4$; subgroup VI).

A group of ANTIGENS not dealt with in the NCI study [5] are the so-called 'minor histocompatibility ANTIGENS' (mHAg; Tab. 3). These ANTIGENS can act as TAA and be instrumental in the rejection of malignant cells after ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (SCT) [45]. mHAg are class I, MHC-presented peptides from polymorphic, naturally processed intracellular proteins that can be encoded by mitochondrial DNA, the Y chromosome or autosomal chromosomes [46]. mHAg-specific $CD8^+$ T cells have been isolated from SCT recipients, particularly those who have developed graft-*versus*-host disease (GVHD) [47]. Twenty-seven mHAg have now been traced back at the molecular level to the proteins from which they originated; 17 are autosomally encoded and 10 are encoded by *H-Y*. Several of these proteins are expressed by malignant cells and/or appear to have a role in malignant cell transformation [48]. Therefore, these mHAg may be

Table 3. Overview of minor histocompatibility antigens [46]

Minor H Antigen & HLA restricting element	Description (gene)	Tissue distribution		Ref.
		General	Details	
Autosomally encoded minor histocompatibility antigens				
HA-1 (A2, B60)	HMHA1 histocompatibility (minor) HA-1	Restricted	Hem, SolTum	[165, 166]
HA-2 (A2)	Myosin 1G	Restricted	Hem	[167]
HA-3 (A1)	AKAP13 A kinase (PRKA) anchor protein 13	Broad		[168]
HA-8 (A2)	KIAA0020	Broad	Hem	[169]
HB-1 (B44)	HMHB1 histocompatibility (minor) HB-1	Restricted	B-ALL, B-LCL	[170]
ACC-1 (A24)	BCL2A1 BCL2-related protein A1	Restricted	Hem, SolTum	[171]
ACC-2 (B44)	BCL2A1 BCL2-related protein A1	Restricted	Hem, SolTum	[171]
UGT2B (A29, B44)	UDP-glucuronosyltransferase 2B17	Restricted	Hem	[172]
LRH-1 (B7)	P2RX5 purinergic receptor P2X	Restricted	Hem, SolTum	[173]
SP110 (A3)	SP110 nuclear body protein	Restricted	Hem	[174]
PANE1 (A2)	Centromere protein M	Restricted	Hem	[175]
C19Orf48 (A2)	Multidrug-resistance related protein on chromosome 19 (open reading frame 48)	Restricted	SolTum	[176]
LB-ECGF (B7)	TYMP thymidine phosphorylase	Restricted	SolTum	[177]
CTSH (A31, A33)	CTSH cathepsin H	Restricted	Hem	[178]
LB-ADIR (A2)	TOR3A torsin family 3, member A	Restricted	Hem, SolTum	[179]
ACC6 (B44)	HMSD histocompatibility (minor) serpin domain	Broad		[180]
CD19 (DQA1*05, DQB1*02)	CD19-encoded mHAgs expressed by B lymphocytes	Restricted	Hem	[181]
HY-encoded minor histocompatibility antigens				
HY-A*0101	Ubiquitin specific peptidase 9, Y-linked	Broad		[182, 183]
HY-A*0201	Lysine (K)-specific demethylase 5D, Y-linked	Broad		[184]
HY-A*3303	Thymosin β4, Y-linked	Broad		[185]
HY-B*0702	Lysine (K)-specific demethylase 5D, Y-linked	Broad		[186]
HY-B*60	Ubiquitously transcribed tetratricopeptide repeat gene	Broad		[187]
HY-DQ5	DEAD box polypeptide 3, Y-linked	Broad		[188]
HY-DRB1*1501	DEAD box polypeptide 3, Y-linked	Broad		[189]
HY-DRB3*0301	RPS4Y1 ribosomal protein S4, Y-linked 1	Broad		[190]
HY-B8	Ubiquitously transcribed tetratricopeptide repeat gene	Restricted	Hem	[191]
HY-B*5201	RPS4Y1 ribosomal protein S4, Y-linked 1	Restricted	Hem, SolTum	[192]

B-ALL, B-acute lymphoblastic leukemia; B-LCL, B-lymphoblastoid cell lines; Hem, hematopoietic tissues; SolTum, solid tumors.

classified as TAA. Genomic typing for mHAg has been standardized by an international collaboration of >30 laboratories worldwide [49]. As mHAg are expressed by normal hematopoietic cells, their use as targets for IMMUNOTHERAPY of cancer is limited to the setting of allogeneic STEM CELL TRANSPLANTATION, in which the patient's hematopoietic system is replaced by that of an HLA-matched, but mHAg-mismatched stem cell donor with the capability to recognize the mHAg expressed by the tumor as foreign. In this setting, mHAg, being allo-ANTIGENS, are not subject to self-TOLERANCE and are likely to be more immunogenic than the above-described MHC-restricted tumor-associated self ANTIGENS and, in this way, constitute a powerful addition to the array of TAA.

Immunotherapy of cancer

Cytokines

CYTOKINES are endogenous molecules that affect the immune response and include, for example, IL-1, IFN-α and TUMOR NECROSIS FACTOR (TNF)-α (with pro-inflammatory properties), IL-2, IL-12 and IFN-γ (with T cell-potentiating properties), IL-8 and macrophage inflammatory protein-1 (with chemotactic properties) and granulocyte-macrophage colony-stimulating factor (GM-CSF, with classical growth-stimulating properties). RECOMBINANT CYTOKINES that have been most widely used against cancer are IFN-α, IL-2 and TNF-α. IFN-α has multiple effects on (tumor) cell proliferation, angiogenesis, immune function and expression of genes coding for MHC class I molecules, tumor ANTIGENS and ADHESION MOLECULES. Its antitumor effects have been well-documented in hairy cell leukemia [50], Kaposi's sarcoma [51] and metastatic renal cell carcinoma [52], while prolonged exposure to IFN-α using the PEGYLATED form of the cytokine led to a significant, sustained reduction of recurrence-free survival in melanoma [53] (see chapter C7).

IL-2 has never been shown to have direct antitumor activity, but has been used for immunostimulation in metastatic renal cell carcinoma and malignant melanoma. Some of these patients obtained longstanding partial (10–15%) or complete (~5%) regression of their tumors; the latter were frequently of long duration (i.e., >7 years), suggesting immunological eradication of the tumor [54]. The diverse multisystem toxicity observed with high-dose i.v. IL-2 therapy has prompted major efforts to develop effective regimens using lower doses of IL-2. While low-dose IL-2 regimens in metastatic melanoma did not appear to be effective, response rates similar to those obtained with high-dose IL-2 therapy have been observed in renal cell carcinoma patients [55] (see chapter C7). A variety of combination regimens has been tested, especially with low-dose IL-2 and IFN-α for renal cell carcinoma. For example, the combination of IL-2, IFN-α and the CYTOTOXIC DRUG 5-fluorouracil has yielded response rates of 2%, 16% and 39% in three major studies [56–58]. This large variation in response rates indicates that there is no convincing evidence that COMBINATION THERAPY is superior to that with IL-2 alone.

TNF-α is a pleiotropic agent with direct and indirect antitumor effects, and is a mediator of septic SHOCK [59]. Hence, dose-limiting toxicity of systemic TNF-α is already encountered at concentrations 10–50 times lower than those needed for antitumor effects in murine models [60]. However, the surgical technique of isolated limb perfusion allows the application of therapeutically effective concentrations of TNF-α; response rates between 64% and 100% have been reported when TNF-α was combined with the CYTOTOXIC DRUG melphalan for the treatment of in-transit melanoma metastases (reviewed in [61]). The combination of TNF-α and melphalan was similarly effective in treating unresectable soft-tissue sarcoma, in which limb amputation could have been avoided in 64–90% of the patients [61]. Animal studies have revealed that the combined use of TNF-α with melphalan rather than the individual compounds is critical to destroy tumors; TNF-α targets the tumor vasculature, allowing strongly enhanced accumulation of melphalan in the tumor, leading to tumor vessel destruction and metabolic shut-down of the tumor [62].

Among the CYTOKINES with high potential for cancer treatment, IL-7, IL-12, IL-15 and IL-21 should be mentioned [63]. IL-7 is a 25-kDa T cell growth factor that costimulates TCR signaling, and is required for

the development and survival of T cells. It signals *via* its RECEPTOR (IL-7R) containing an α and a γ chain. IL-7Rα+ T cells are bound and become memory cells. Treatment of cancer patients with IL-7 in phase I/II trials did not show toxicity, but revealed significant increments in – mainly naïve – $CD4^+$ and $CD8^+$ T cells [64, 65]. There appeared to be a preferential expansion of naïve T cells with an increased TCR diversity. Thus, IL-7 may serve as an adjuvant to enhance the EFFICACY of tumor vaccines to counteract self-TOLERANCE.

IL-12 is a 70-kDa glycoprotein that binds IL-12R on NK cells, T cells, DC and MACROPHAGES [66]. It promotes IFN-γ release by these cells and induces Th1 polarization as well as proliferation of IFN-γ-expressing T cells. Phase I/II trials in melanoma and renal cell carcinoma have shown low EFFICACY of IL-12 monotherapy, but IL-12 may be more effective when co-administered with a vaccine ito polarize Th1 responses and augment $CD8^+$ T cell responses.

IL-15 is produced by MACROPHAGES and DC and acts on $CD4^+$ and $CD8^+$ T cells as well as NK cells [67]. IL-15Rα presents IL-15 to the IL-2/15 β RECEPTOR and the γ chain RECEPTOR. IL-15 inhibits antigen-induced cell death, reverses T cell anergy, substitutes $CD4^+$ T cell help in CTL induction and induces long-lived $CD8^+$ T cells with potent antitumor cell activity.

IL-21 belongs to the γ-chain family of CYTOKINES and is primarily produced by $CD4^+$ T cells and induces differentiation of pro-inflammatory $CD4^+$ Th17 cells, stimulates memory and naïve $CD8^+$ T cell expansion synergistically with IL-7 or IL-15, and improves expansion and AFFINITY of antigen-specific T cell CLONES [68]. In this way, IL-21 may be critical for *in vitro* generation and expansion of tumor antigen-specific CTL in ADOPTIVE IMMUNOTHERAPY.

Monoclonal antibodies

Initially, mAb were produced from murine hybridomas produced by fusion of B LYMPHOCYTES from immunized mice with murine myeloma cells ("murine mAb") [69]. However, the early clinical applications of such mAb against cancer were hampered by the development of human antimurine Ab (HAMA) responses that rendered these mAb ineffective by neutralization and shortened their *in vivo* survival. Consequently, techniques were developed (i) to combine the antigen-binding parts of the mouse ANTIBODY with the effector parts of a human ANTIBODY (CHIMERIC mAb); (ii) to construct such molecules with only complementarity-determining regions derived from a mouse, and the remainder of the variable regions, and constant regions, derived from a human source (HUMANIZED mAb); or (iii) to make HUMAN MAB derived entirely from a human source, transgenic mice, phage display, human hybridomas, or EBV-immortalized human B lymphocyte cell lines. The HUMAN MAB are the least immunogenic, but the engineered mAb have the advantages of reduced immunogenicity, improved half-life and optimized SPECIFICITY [70]. Table 4 provides an overview of marketed mAb approved by the United States Food and Drug Administration (FDA) that are currently available for the treatment of cancer.

The mechanism of action of mAb against tumors is complex and highly dependent on the nature of the TARGET molecule. The effects of mAb can be enhanced by combination of cytoreductive treatment or CYTOKINES. mAb can activate effector functions through their Fc portions: ANTIBODY-dependent cellular CYTOTOXICITY (ADCC, in which the mAb interact *via* their Fc portions with the Fc RECEPTORS on phagocytic cells and NK LYMPHOCYTES) and complement-dependent CYTOTOXICITY (in which the Fc RECEPTORS bind components of the complement cascade). For example, ADCC appears to be the main effector mechanism for rituximab to eliminate B cells [71]. In addition, mAb may also induce APOPTOSIS, cell cycle arrest, inhibition of cell proliferation, angiogenesis and metastatic spread. A case in point is trastuzumab whose mechanism of action includes, besides activation of the IMMUNE SYSTEM, down-regulation of the constitutive growth signaling properties of the Her-2 RECEPTOR network on cancer cells [72]. Finally, anticancer mAb may be covalently linked to drugs in order to selectively deliver these to tumors with the aim of improving antitumor EFFICACY and reduce the systemic toxicity of therapy. Examples of such immunoconjugates are tositumomab conjugated with radioactive iodine for B-NHL, and gemtuzumab ozogamicin, a CD33 mAb conjugated with

Table 4. FDA-approved marketed mAb for treatment of cancer

Generic name	Structure	Target	Clinical indication	Reference
Rituximab	Chimeric	CD20	NHL	[193]
Trastuzumab	Humanized	Her2	mBC	[194]
Bevacizumab	Humanized	VEGF	mCRC NSCLC Her2-BC	[195] [196] [197]
Cetuximab	Chimeric	EGFR	SCCHN	[198]
Tositumomab	Murine	CD20	NHL	[199]
Alemtuzumab	Humanized	CD52	B-CLL	[200]
Gemtuzumab ozogamicin	Humanized	CD33	AML	[73]
Panitumumab	Human	EGFR	mCRC	[201]
Ibritumomab tiuxetan	Murine	CD20	NHL	[199]

AML, acute myeloid leukemia; B-CLL, B-chronic lymphocytic leukemia; EGFR, epidermal growth factor receptor; (m) BC, (metastatic) breast cancer; mCRC, metastatic colorectal cancer; NHL, non-Hodgkin's lymphoma; NSCLC, non-small cell lung cancer; SCCHN, squamous cell carcinoma of the head and neck; VEGF, vascular endothelial growth factor.

calicheamicin that cleaves double-stranded DNA in acute myelogenous leukemia cells [73].

Cancer vaccination

None of the 75 ANTIGENS that had been addressed by the NCI study [5] fulfilled all of the characteristics of an "ideal cancer antigen." The cumulative scores in the ranking system used by the NCI task force ranged from 0.81 to 0.13. On the basis of cumulative scores the top 10 ANTIGENS were WT1, MUC-1, LMP2, HPV E6 E7, EGFRVIII, Her2/neu, IDIOTYPE, MAGE A3, p53 non-mutant and NY-ESO-1. Therapeutic function and immunogenicity were considered the most important factors and represented almost half of the overall cumulative score for candidate vaccine ANTIGENS. For therapeutic function, 20 ANTIGENS were considered to have at least fair data for effectivity in controlled vaccine trials (i.e., score 0.59 or higher; Tab. 2). For immunogenicity, 46 of the 75 ANTIGENS had documented immunogenicity in clinical trials [i.e., (maximum) score 1.0]. However, none of these ANTIGENS have yet been approved by FDA for general use.

Adoptive autologous cellular immunotherapy

Based on promising animal data, initial approaches to adoptive cellular IMMUNOTHERAPY of cancer have made use of *ex vivo* IL-2-activated peripheral blood mononuclear cells that exhibited so-called lymphokine-activated killer (LAK) activity [74]. LAK activity was defined as the capability to kill fresh and cultured tumor cells, but not normal cells, in an MHC-unrestricted fashion. Patients with advanced melanoma and renal cell cancer were treated with high-dose IL-2 and up to 2×10^{11} autologous LYMPHOCYTES with LAK activity. Although complete and partial tumor regressions were seen in up to one third of patients [75], subsequent randomized studies attributed these responses to IL-2, while no evidence of a benefit of LAK cells was demonstrated [76, 77].

In the long run, some 5% of patients treated with IL-2 for metastatic renal cell cancer or melanoma achieved long-term remissions [11, 78]. The mechanism behind these remissions is still unknown. The immunotherapeutic intervention with IL-2 may have interfered with negative immune-regulatory pathways between tumor and host as discussed above.

Subsequently, most clinical research on adoptive autologous cellular IMMUNOTHERAPY has focused on melanoma. This choice was based on the relatively frequent finding of tumor-specific T cells in such patients, the large number of melanoma-encoded TAA, the molecular characterization of melanoma-specific TCR, and the fact that the disease is often resistant to standard chemotherapy [79]. *Ex vivo* IL-2-cultured TIL yielded a higher response rate (34%), although only of short duration [11]. In subsequent trials, the following observations were made: (i) administration of *ex vivo* generated HLA-restricted, melanoma-specific T cells by cloning resulted in relatively short survival of the infused cells and only modest clinical response rates [80]; (ii) following intensified cytoreductive pretreatment, administration of T cells that were only partially selected for SPECIFICITY resulted in better T cell survival in terms of improved response rates and duration of responses [8]; and (iii) intense preconditioning resulted in the highest response rates (>70%) with proven durability in some patients [81]. The beneficial effect of immunosuppressive pretreatment is most likely due to elimination of negative immunoregulatory mechanisms in the patients that interfere with the antitumor effects. The replicative history of the T cells that were used in the latter studies was shorter (i.e., featuring long telomere lengths and high CD27 expression). These trials, along with the supporting preclinical data, provide the current paradigm for the use of adoptive cell transfer, in which infusion of – preferably "young" – cells is combined with preconditioning chemotherapy. In addition, the use of minimally selected, bulk-cultured melanoma-derived TIL confirmed that a response rate of 50% could indeed be achieved [82].

To overcome the disadvantage of isolating and expanding TIL, a more universal strategy has been developed to generate tumor-specific T cells: TCR GENE THERAPY [83, 84]. Two basic approaches have been developed to engineer T cells: (i) the transfer of genes derived from NATURAL TCR [85]; and (ii) the transfer of genes coding for CHIMERIC RECEPTORS typically containing an ANTIBODY fragment linked to a T cell signal-transducing domain [86]. Initial clinical experiences have not only revealed the potential power but also the potential dangers of this approach. As for the latter, both studies showed so-called "on-TARGET" toxicity, i.e., damage of healthy cells that shared the TARGET antigen with the tumor cells, i.e., carbonic anhydrase IX on renal cell cancer [85], and MART-1 and gp100 on melanoma [86]. Although such toxicities may be suppressed, they strongly suggest that T cell therapy should be directed against "safer" TARGET ANTIGENS, i.e., oncofetal ANTIGENS or those with absolute SPECIFICITY (Tab. 1).

Allogeneic hematopoietic stem cell transplantation

High-dose chemoradiotherapy followed by rescue from the resulting ablation of hematopoietic function with SCT from an HLA-matched donor has become standard therapy for many hematological malignancies. One problem with this treatment is GVHD due to donor-derived T cells recognizing mismatched mHAg expressed by normal tissues of the host (Tab. 3). As the malignant cells that survive chemoradiotherapy are also of host origin, patients who develop GVHD have less frequent recurrence of original disease due to the associated graft-*versus*-tumor (GVT) effect. T cells mediate this antitumor activity, because (i) infusions of T cells from the SCT donor to treat leukemic relapse after SCT sometimes result in complete remissions, and (ii) the complete remissions observed after so-called non-myeloablative SCT must result from GVT effects because the reduced-intensity cytoreductive therapy primarily serves as IMMUNOSUPPRESSION, and is unable to eliminate all residual disease [87]. The development of non-myeloablative conditioning regimens prior to allo-SCT has allowed this therapy to be used in elderly and disabled patients, including those with solid tumors. A review of >1000 patients under the auspices of the European Group for Blood and Marrow Transplantation (EBMT) highlighted allogeneic SCT

as an emerging treatment modality for solid tumors [88]. Most experience has been gained on renal cell cancer (12 studies, 335 patients) and breast cancer (7 studies, 143 patients). For both diseases, proof of concept for a GVT effect was considered to exist, while for other tumors (colorectal, ovarian, pancreatic) the existence of such an effect was suggested. Antitumor responses were generally associated with acute and chronic GVHD, emphasizing the relatively high toxicity of this form of treatment. As a result, allogeneic SCT for solid tumors should be considered still experimental until definitive proof of a clinical benefit has been achieved in ongoing studies.

Conclusions and future developments

It has now (firmly) been established that the human IMMUNE SYSTEM is capable of recognizing and spontaneously eliminating arising tumors, although cancer cells are generally less immunogenic than microbial pathogens such as bacteria, fungi and viruses. Nevertheless, immunotherapeutic modalities, such as CYTOKINES and mAb, have already become components of several standard treatment regimens of human malignancies. Many vaccines have advanced through preliminary testing to EFFICACY trials, and have shown little toxicity but, in general, also have limited effects in patients with established tumors. Tumors frequently interfere with the development and function of immune responses. Thus, one of the challenges for cancer IMMUNOTHERAPY is to use advances in cellular and molecular immunology to develop strategies that effectively and safely augment antitumor responses. With our increasing understanding of the requirements for immune cell activation, homing and accumulation at tumor sites, and of the disruption of regulatory mechanisms that inhibit immunological anticancer responses at the sites of the tumors, our abilities to design and engineer immunotherapeutic approaches with antitumor capacities beyond what can be elicited from the normal IMMUNE SYSTEM, is advancing.

Initial clinical trials of adoptive transfer of large numbers of autologous tumor-reactive T LYMPHOCYTES (TIL) have shown antitumor effects and, in particular, against melanoma. The EFFICACY of this form of adoptive CELLULAR THERAPY was significantly enhanced in combination with IMMUNOSUPPRESSION. The ability to transfer genes with high efficiency into polyclonally activated T cells has raised wide interest in the genetic retargeting of T LYMPHOCYTES against tumors. Initial clinical experience with this approach has shown its feasibility. However, this form of treatment is still in its infancy due to the potential of the engineered T cells to cause "on-TARGET" toxicity, and their generally short persistence. Therefore, most current clinical trials of ADOPTIVE IMMUNOTHERAPY are based on the use of minimally cultured TIL. The transfer of TAA-specific RECEPTORS to HEMATOPOIETIC STEM CELLS (HSC), to retarget their progeny against cancer, might appear to be a solution to this problem, but the observation of oncogenic events in 4 of 9 patients after transfer of a growth factor RECEPTOR-encoding gene into HSC [89] has tempered the enthusiasm for this approach. Transplantation of allogeneic HSC has been shown to induce long-lasting remission (up to tens of years) in patients with leukemia, and (following pretreatment with a reduced-intensity cytoreductive regimen) might also be of value in older patients with solid tumors, although still associated with significant toxicity in such patients [14].

Selected readings

Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, Mellman I, Prindiville SA, Viner JL, Weiner LM, Matrisian LM (2009) The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res* 15: 5323–5337

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Important websites

The ATTACK consortium on adoptive immunotherapy using engineered T cells: *www.attach-cancer.org*

European Group for Blood & Marrow Transplantation: *www.ebmt.org*

Human Cell Differentiation Molecules is an organisation which runs the Human Leucocyte Differentiation Antigens Workshops, and names and characterises CD molecules: *www.hdcm.org*

Minor histocompatibility knowledge database: *www.lumc.nl/rep/cod/redirect/5033/dbminor*

T cell recognition of tumor-associated antigens: epitope prediction of HLA-resticted antigens: *www.syfpeithy.de*

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Neuroimmunoendocrinology

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Historical background

Neuroimmunoendocrinology refers to the study of the interactions among behavioral, neural, neuroendocrine, and immunological processes of adaptation. Although relationships between the brain and the IMMUNE SYSTEM had been suggested for many years, research mainly during the last 30 years has provided mechanisms for how these systems may interact. The current interest in neuroimmunoendocrinology is sustained by the now widely held belief that it represents a bidirectional system. The nervous system not only influences immune function, but the latter modifies the nervous system. In one of the earliest studies, Metal'nikov and Chorine in 1926 showed that repeated exposure of guinea pigs to antigenic material (unconditioned stimuli) paired with scratching (conditioned stimuli) resulted in the IMMUNE SYSTEM responding to scratching alone [1]. By early 1940, Alexander and French [2] showed that psychosomatic pathology was connected to repressed emotions. Selye discovered the role of steroid hormones in regulating immunity and contributed to our understanding of MAST CELLS in pathological phenomena [3]. A few years later, Szentivanyi and colleagues reported that lesions of the hypothalamus inhibited ANTIBODY (Ab) production and the anaphylactic reaction in rabbits and guinea pigs [4]. This was followed by a study showing that lesions in the anterior hypothalamus of rabbits prevented the formation of complement-fixing ANTIBODIES [5]. In the 1960s, Solomon [6] concluded from his studies that psychological well-being served a protective function against an AUTOIMMUNE DISEASE. In another important early observation, Ader [7] showed that avoidance conditioning could increase virus infection and that psychological factors could alter the onset and course of AUTOIMMUNE DISEASE. Additional results identifying specific neurohormone RECEPTORS on cells of the IMMUNE SYSTEM, evaluating brain lesions and identifying nerve fibers in compartments of LYMPHOID ORGANS along with the biological relevance of innervation on immune function supported the idea of a very dynamic interaction between the immune and nervous system [8]. The studies of David and Suzanne Felton showed that noradrenergic nerve fibers distribute into specific zones of T cells and MACROPHAGES, including sites of entry and exit, antigen presentation and lymphocyte activation [9]. Over the same time frame, a number of reports appeared indicating the efficacious effects on the IMMUNE SYSTEM of hormone therapy in hormone-deficient animals [10]. Thus, it became apparent that the neuroendocrine system could interact with and modulate the IMMUNE SYSTEM by the release of hormones.

Research into the reciprocal situation in which the stimulation of the IMMUNE SYSTEM altered CENTRAL NERVOUS SYSTEM (CNS) function firmly established the bidirectional nature of the communication between these two systems. Initially, it was shown that the IMMUNE SYSTEM as a consequence of antigenic challenge altered the firing rate of hypothalamic neurons [11]. Supernatant fluids from activated LEUKOCYTES could mimic this phenomenon, and it is now clear that a wide range of lymphocyte products influence the synthesis and secretion or release of neuroendocrine hormones and neurotransmitters [12]. Our studies, reviewed in part below, initially showed that cells of the IMMUNE SYSTEM could be a source of pituitary hormones and that immune-derived CYTOKINES could function as hormones and hypothalamic-releasing factors [12]. Our discovery that cells of the IMMUNE SYSTEM produce PRO-OPIOMELANOCORTIN (POMC) peptides grew out of the original observa-

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tion that interferon (IFN)-α had analgesic effects. We now know that there are distinct domains in the IFN-α molecule that mediate immune and analgesic effects, and that the opioid-like analgesic effects of IFN-α are mediated *via* the μ-opioid RECEPTOR [13]. Further studies suggested a mechanism by which the body's two principle recognition organs, the brain and the IMMUNE SYSTEM, may influence each other, i.e., speak the same biochemical language. Collectively, these relationships provide the foundation for behaviorally induced alterations in immune function and for immunologically based changes in behavior. Accumulating *in vitro* and *in vivo* studies indicate that shared ligands and RECEPTORS are used as a common chemical language within and between the immune and neuroendocrine systems. Specifically, physical and psychological stimuli stimulate the release of neurotransmitters, hormones, and CYTOKINES, which bind to RECEPTORS on cells of the IMMUNE SYSTEM and alter their function (Fig. 1). In addition, the IMMUNE SYSTEM converts recognition of non-cognitive stimuli, such as viruses and bacteria, into information in the form of CYTOKINES, peptide hormones, and neurotransmitters, which act on RECEPTORS in the neuroendocrine system to alter its function. Soluble products that appear to transmit information from the immune compartment to the CNS include thymosins, lymphokines [INTERLEUKIN (IL)-1, -2, -6, TUMOR NECROSIS FACTOR (TNF-α) and IFN], ADRENOCORTICOTROPIN (ACTH), and opioid peptides [14]. The predominant effects of these CYTOKINES are to stimulate the HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS and suppress the HYPOTHALAMIC-PITUITARY-THYROID (HPT) AXIS, HYPOTHALAMIC-PITUITARY-GONADAL (HPG) AXIS, and GROWTH HORMONE (GH) release. The findings suggest an important immunoregulatory role for the brain and an important sensory function for the IMMUNE SYSTEM [15]. This chapter briefly describes the basic and clinical evidence for the role that neuroendocrine hormones play in communication between the immune and neuroendocrine system.

Regulation of the immune system by neuroendocrine hormones

A large amount of evidence exists to support both the presence of RECEPTORS for neuroendocrine hormones on cells of the IMMUNE SYSTEM, as well as the ability of these hormones to modulate specific func-

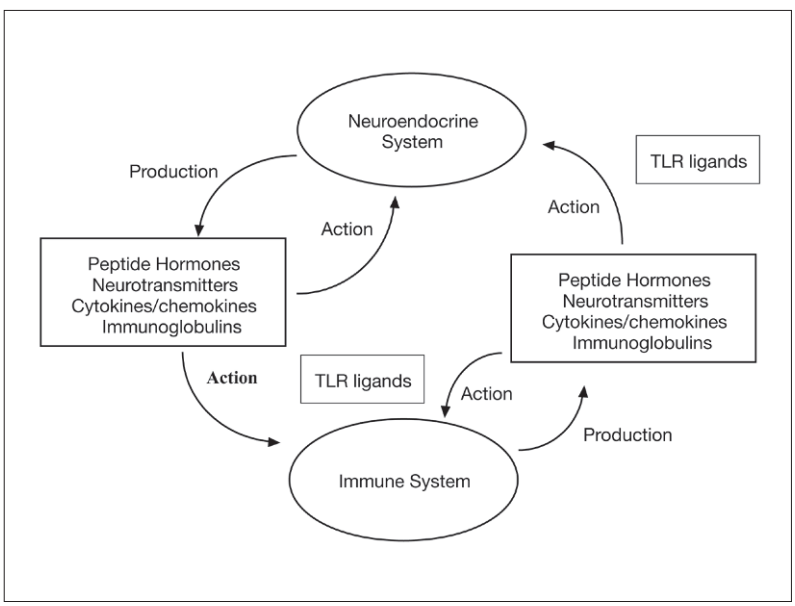

FIGURE 1
A molecular communication circuit within and between the immune and neuroendocrine systems involving shared ligands and their receptors. (Reprinted with permission from Weigent and Blalock [12] by The Society for Leukocyte Biology.)

Table 1. Modulation of immune responses by neuroendocrine hormones

Hormone	Cell type or tissue with hormone receptor	Modulating effects
Corticotropin	Rat spleen T and B cells	Antibody synthesis IFN-γ production B lymphocyte growth
Endorphins	Spleen	Antibody synthesis Mitogenesis Natural killer cell activity
MSH	Lymphocytes, monocytes	Down-regulation of proinflammatory cytokines, adhesion molecule expression and NO production
Thyrotropin	Neutrophils, monocytes, B cells	Increased antibody synthesis Co-mitogenic with ConA
GH	PBL, spleen, thymus	Cytotoxic T cells Mitogenesis
LH and FSH	–	Proliferation Cytokine production
PRL	T and B cells	Co-mitogenic with ConA Induces IL-2 receptors
CRH	PBL	IL-1 production Enhanced natural killer cell activity Immunosuppressive
TRH	T cell lines	Increased antibody synthesis
GHRH	PBL and spleen	Stimulates proliferation
SOM	PBL	Inhibits natural killer cell activity Inhibits chemotactic response Inhibits proliferation Reduces IFN-γ production

tions of the various immune cell types (Tab. 1; [12]). Several examples of hormones are discussed below and the reader is directed to review articles for a further discussion of the topic.

Actions of adrenocorticotropin and endorphins

The RECEPTORS for ACTH and ENDORPHINS have been identified on cells of the IMMUNE SYSTEM as well as the ability of these hormones to modulate many aspects of immune reactivity. The findings show that B LYMPHOCYTES contain three times the number of ACTH-binding sites compared with T cells, and that treatment with a MITOGEN increases the number of high-AFFINITY sites two- to threefold on both cell types. Monospecific antiserum to the ACTH RECEPTOR on Y-1 adrenal cells recognizes the ACTH RECEPTOR on LEUKOCYTES. The binding of ACTH initiates a signal transduction pathway that involves both cAMP and mobilization of Ca^{2+}. More recent analysis of the effects of ACTH by patch-clamp methods suggests that this hormone can modulate macrophage functions through the activation of Ca^{2+}-dependent K^+

channels [16]. ACTH has been shown to suppress MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) class II expression, stimulate NATURAL killer (NK) cell activity, suppress IFN-γ production, modulate IL-2 production, and function as a late-acting B cell growth factor.

The production of opioid peptides in immune cells [17] and lymphocyte RECEPTORS for the opioid peptides have also been studied and found to share many of the features, including size, sequence, immunogenicity and intracellular signaling, of those described on neuronal tissue. Many aspects of immunity are modulated by the opiate peptides (Tab. 2). The mechanisms by which opiate peptides influence such diverse activities remain unclear. However, it is known that β-endorphin alters immune cell calcium ion flux while shutting down K^+ channel function [18]. Thus, opiates may bind both the classical opioid RECEPTORS and K^+ channels to modulate immune cell activity [19].

Additional opioid peptides, other than ENDORPHINS and enkephalins, located in the mammalian nervous system and immune tissues are endomorphins (EM) [20]. EM are tetrapeptides that show analgesic properties in animal models of neuropathic or neurogenic pain, but also can exert potent anti-inflammatory effects in both acute and chronic peripheral INFLAMMATION [21]. Immune cell-derived and exogenously applied EM alleviate prolonged inflammatory pain through selective activation of peripheral opioid RECEPTORS [22]. However, not all the biological activity of EM appears to require the opioid RECEPTOR, as the suppression of the *in vitro* Ab formation of mouse spleen cells by EM was not blocked by opioid RECEPTOR antagonists [23].

The anti-inflammatory influences of α-melanocyte-stimulating hormone (MSH) and other melanocortins are primarily exerted through inhibition of inflammatory mediator production and cell migration [24]. These effects occur through binding of melanocortins to melanocortin RECEPTORS on cells of the IMMUNE SYSTEM and also *via* descending anti-inflammatory neural pathways induced by stimulation of α-MSH RECEPTORS within the brain [25]. Almost all of the cells responsive to melanocortins, including MACROPHAGES, LYMPHOCYTES, NEUTROPHILS, DENDRITIC CELLS, astrocytes and microglia, express the melanocortin type-1 RECEPTORS. The *in vitro* and *in vivo* inhibitory effects of α-MSH influence adhesion, production of CYTOKINES and other mediators of INFLAMMATION, including IL-1, IL-6, IL-10, TNF-α, CHEMOKINES, and NITRIC OXIDE (NO) [24]. Various skin cells are also the source and TARGET for the anti-inflammatory effects of MSH [26]. The broad effects of α-MSH on inflammatory mediator production is thought to occur through the participation of G proteins, the Janus kinases/signal transducer activator of transcription (JAK/STAT) pathway, and inhibition of the activation of the NUCLEAR FACTOR NF-κB.

Actions of growth hormone and prolactin

It has been clearly shown that cells of the IMMUNE SYSTEM contain RECEPTORS for GH and prolactin (PRL) and that these hormones are potent modulators of the immune response [27]. The PRL and GH RECEPTORS have been shown to be members of the superfamily of cytokine RECEPTORS involved in the growth and differentiation of hematopoietic cells.

Table 2. Modulation of immune responses by opiates

1. Enhancement of the natural cytotoxicity of lymphocytes and macrophages toward tumor cells
2. Enhancement or inhibition of T cell mitogenesis
3. Enhancement of T cell rosetting
4. Stimulation of human peripheral blood mononuclear cells
5. Inhibition of major histocompatibility class II antigen expression

A systematic survey of PRL RECEPTOR expression by FLOW CYTOMETRY showed that PRL RECEPTORS are universally expressed in normal hematopoietic tissues with some differences in density, which could be increased by concanavalin (Con)A treatment *in vitro* and exercise *in vivo*.

GH RECEPTORS from a number of species have been sequenced and the co-crystallization of human GH with the GH RECEPTOR has been achieved. GH binding and cellular processing of the GH RECEPTOR has been studied in a cell line of immune origin. In the IM-9 cell line, it has been shown that GH stimulates proliferation and that the GH RECEPTOR can be down-regulated by phorbol esters. Several lines of evidence indicate that activation of the GH RECEPTOR increases tyrosine kinase activity, and that the GH RECEPTOR is associated with a tyrosine kinase in several cell types, including the IM-9 cell. A role for GH in immunoregulation has been demonstrated *in vitro* for numerous immune functions (Tab. 3; [27]). It is not clear whether GH directly influences intrathymic or extrathymic development, or acts indirectly by augmenting the synthesis of thymulin or insulin-like growth factor-1 (IGF-1). These observations suggest that GH may stimulate local production of IGF-1, which acts to promote tissue growth and action in a paracrine fashion.

The potential effect of GH in tumorigenesis, particularly in acute leukemia, is controversial. An active area of research over the past several years has been the immune-enhancing effects of both SYNTHETIC (hexarelin) and NATURAL (GHrelin) GH secretatogues. Their primary effect on cells of the IMMUNE SYSTEM appears to be promotion of cell division. A possible role for lymphocyte GH, through an increase in synthesis and secretion, has been suggested in the mechanism of cell proliferation. Likewise, PRL can have modulating effects on the IMMUNE SYSTEM [27]. Data show that suppression of PRL secretion in mice with bromocriptine increases the lethality of a *Listeria* challenge and abrogates T cell-dependent activation of MACROPHAGES. Abs to the PRL RECEPTOR have been shown to abolish PRL-induced proliferation of Nb2 cells. More recent studies suggest that PRL may promote survival of certain lymphocyte subsets, modulate the naïve B cell REPERTOIRE, and promote antigen-presenting functions [28]. The lymphocyte source of PRL may explain the association of hyperprolactinemia with AUTOIMMUNE DISEASES.

Actions of hypothalamic releasing hormones

In addition to pituitary hormones, HYPOTHALAMIC RELEASING HORMONE RECEPTORS and their effects have been documented on cells of the IMMUNE SYSTEM. A number of similarities have been identified between the pituitary and spleen binding of CORTICOTROPIN RELEASING HORMONE (CRH), including AFFINITY and apparent subunit molecular weight. Although there are multiple species of CRH RECEPTORS, the major two, CRH-R1 and CRH-R2, are both found on LEUKOCYTES [29]. *In vitro*, CRH enhances T and B cell mitogenesis and cytokine induction, and inhibits phagocyte activation, Ab production, and NK activity [30]. In vivo. although CRH is generally proinflammatory, it may inhibit NK cell activity and Ab production. The inhibitory activities *in vivo* are thought to be mediated through the actions of GLUCOCORTICOIDS, whereas the

TABLE 3. MODULATION OF IMMUNE FUNCTIONS BY GROWTH HORMONE

1. Stimulation of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis in the spleen and thymus
2. Stimulation of neutrophil differentiation and thymic development
3. Stimulates erythropoiesis
4. Influences the activity of cytolytic cells, including T lymphocytes and NK cells
5. Stimulates the production of superoxide anion from macrophages

direct effects are proinflammatory [30]. The effects of ACTH and ENDORPHINS discussed earlier may be initiated in the IMMUNE SYSTEM *via* the production of these hormones by cells of the IMMUNE SYSTEM in response to CRH (see below).

The GROWTH HORMONE RELEASING HORMONE (GHRH) RECEPTOR has also been identified on cells of the IMMUNE SYSTEM. The GHRH RECEPTOR binding sites are saturable and are found on both thymocytes and splenic LYMPHOCYTES. After GHRH binding to its RECEPTOR, there is a rapid increase in intracellular Ca^{2+}, which is associated with the stimulation of lymphocyte proliferation. Other *in vitro* findings suggest that GHRH may inhibit NK cell activity and CHEMOTAXIS, and increase IFN-γ secretion. Locally produced GH has been suggested to play a role in tumor development and/or tumor progression [31]. A variety of malignancies, including bone tumors, glioblastomas, lung carcinomas, prostatic, renal, pancreatic, colorectal and ovarian cancers can be inhibited by GHRH antagonists [32]. In a number of tumor cell lines, GHRH antagonists were shown to inhibit the autocrine production of IGF-2 and decrease cell proliferation both *in vitro* and *in vivo* [33, 34]. Thus, GHRH antagonists may directly inhibit intratumor IGF-1 and/or IGF-2 expression by blocking the GH-axis autocrine growth loop. Our own data support [35, 36] this model and, in addition, strongly suggest that endogenous GH is involved in the acquisition of IGF responsiveness, which may be an important event during malignant transformation. Recently, it has been shown that GHRH may play a critical role in the development of experimental autoimmune encephalomyelitis (EAE) in mice [37]. Splenocytes from myelin oligodendrocyte glycoprotein (MOG)-immunized mice deficient in the GHRH RECEPTOR (C57BL/6J-GHRHR$^{lit/lit}$) proliferated normally in response to MOG peptide; however, the animals were resistant to MOG-induced EAE. Another GH secretogogue, ghrelin, secreted from mucosal endocrine cells of the stomach but distributed in lymphoid tissues, has also been found to suppress EAE [38]. The suppression of EAE was accompanied by reduced mRNA levels of proinflammatory CYTOKINES such as TNF-α, IL-1β, and IL-6 in microglia at the peak of disease, suggesting a role for ghrelin as an anti-inflammatory hormone.

LEUKOCYTES have also been shown to respond to THYROTROPIN-releasing hormone (TRH) treatment by producing THYROTROPIN (TSH) mRNA and protein. Recent work has shown the presence of two RECEPTOR types for TRH on T cells. One of these sites satisfies the criteria for a classical TRH RECEPTOR and is involved in the release of IFN-γ from T cells. TRH at very low concentrations enhances the plaque-forming cell response *in vitro via* production of TSH. In this instance, T cells were shown to produce TSH, while other studies suggest that DENDRITIC CELLS and MONOCYTES may also produce biologically active TSH [39]. Interestingly, T LYMPHOCYTES cultured with T3 and T4, but not TSH or TRH, showed enhanced APOPTOSIS with reduced expression of B cell lymphoma-2 (Bcl-2) protein. The TRH peptide precursor has been reported in the spleen, but the cell type producing this peptide and its mechanism of secretion are yet to be determined.

The existence of distinct subsets of SOMATOSTATIN (SOM) RECEPTORS on the Jurkat line of human leukemic T cells and U266 IgG-producing human myeloma cells has also been described. The authors speculate that two subsets of RECEPTORS may account for the biphasic concentration-dependent nature of the effects of SOM in some systems. Although GH and PRL have immunoenhancing capabilities, SOM has potent inhibitory effects on immune responses. SOM has been shown to significantly inhibit Molt-4 lymphoblast proliferation and phytohemagglutinin (PHA) stimulation of human T LYMPHOCYTES, and nanomolar concentrations are able to inhibit the proliferation of both spleen-derived and PEYER'S PATCH-derived LYMPHOCYTES. Other immune responses such as superantigen-stimulated IFN-γ secretion, ENDOTOXIN-induced leukocytosin, and colony-stimulating activity release, are also inhibited by SOM.

Neuroendocrine hormone release by immune system cells

There is now overwhelming evidence that cells of the IMMUNE SYSTEM also produce neuroendocrine hormones. This was first established for ACTH and subsequently for TSH, GH, PRL, luteinizing hormone (LH), follicle stimulating hormone (FSH), and the

Table 4. List of neuroendocrine hormones released by cells of the immune system [12]

Pituitary hormones	Hypothalamic hormones
Adrenocorticotropin	Somatostatin
Thyrotropin releasing hormone	Corticotropin-releasing hormone
Growth hormone	Growth hormone-releasing hormone
Prolactin	Luteinizing hormone-releasing hormone
Luteinizing hormone	
Follicle-stimulating hormone	

hypothalamic hormones, SOM, CRH, GHRH, and LH-releasing hormone (LHRH) (Tab. 4; [12]). The evidence strongly supports the notion that neuroendocrine peptides and neurotransmitters, endogenous to the IMMUNE SYSTEM, are used for both intra-IMMUNE SYSTEM regulation, as well as for BIDIRECTIONAL COMMUNICATION between the immune and neuroendocrine systems. Although initially controversial, it is now clearly established that cells of the IMMUNE SYSTEM produce ACTH. The evidence is overwhelming and includes complete identity of the amino acid sequence of the ACTH peptide and nucleotide sequence of a cDNA from reverse transcription-polymerase chain reaction (RT-PCR) of lymphocyte mRNA to pituitary ACTH [30]. Leukocyte ACTH induces mouse Y-1 adrenal cells to round up morphologically and to secrete corticosteroids, and can be induced by viruses, bacterial products, and CRH. Interestingly, CRH not only induces leukocyte POMC, but this hormone is also produced by LEUKOCYTES. The CRH protein appears to be similar to hypothalamic CRH but not identical [40]. Stressors such as hypoxia, hypothermia, and hyperosmolarity induce LYMPHOCYTES to secrete CRH [41]. The studies show that, although the structure of these peptides are identical to those identified in the neuroendocrine system, both similarities and differences may exist in the abundance of particular transcripts and in the mechanism of synthesis to the patterns previously described in the neuroendocrine system.

At least two possibilities exist concerning the potential function of these peptide hormones produced by the IMMUNE SYSTEM. First they act on their classic neuroendocrine TARGET tissues. Second, they may serve as endogenous regulators of the IMMUNE SYSTEM. With regard to the latter possibility, it is clear that neuroendocrine peptide hormones can directly modulate immune functions. These studies, however, do not specifically address endogenous as opposed to exogenous regulation by neuroendocrine peptides. A number of investigators have now been able to demonstrate that such regulation is endogenous to the IMMUNE SYSTEM. Specifically, TSH is a pituitary hormone that can be produced by LYMPHOCYTES in response to TRH and, like TSH, TRH enhanced the *in vitro* Ab response [42]. The enhancement was not observed with GHRH, arginine vasopressin (AVP), or LHRH and was blocked by Abs to the β subunit of TSH. Thus, it appears that TRH specifically enhances the *in vitro* Ab response *via* production of TSH. This was the first demonstration that a neuroendocrine hormone (TSH) can function as an endogenous regulator within the IMMUNE SYSTEM.

A large number of human hematopoietic cell lines and tumors synthesize and release PRL [43]. The evidence suggests a low constitutive level of PRL expression inducible by IL-2 and inhibited by dexamethasone. In T cells, PRL is translocated to the nucleus in an IL-2-dependent P13 kinase pathway inhibited by rapamycin. Immune cell-derived PRL most likely plays a role in hematopoietic cell differentiation and proliferation. In another study, Ab to PRL was shown to inhibit mitogenesis through neutralization of the lymphocyte-associated PRL.

Furthermore, coordinate GENE EXPRESSION of LHRH and LHRH RECEPTOR has been shown in the Nb2 T cell line after PRL stimulation [44].

Two different approaches have provided convincing evidence that endogenous neuroendocrine peptides have autocrine or paracrine immunoregulatory functions. First, an opiate antagonist was shown to indirectly block CRH enhancement of NK cell activity by inhibiting the action of immunocyte-derived opioid peptides. Second, we have used an antisense oligodeoxynucleotide (ODN) to the translation start site of GH mRNA to specifically inhibit leukocyte production of GH. The ensuing lack of GH resulted in a marked diminution in basal rates of DNA synthesis in such antisense ODN-treated LEUKOCYTES, which could be overcome by exogenously added GH [45]. Another group examining SOM found that antisense ODN to SOM dramatically increased lymphocyte proliferation in culture [46]. Additionally, LHRH agonists were found to diminish NK cell activity, stimulate T cell proliferation, and increase IL-2 RECEPTOR expression, suggesting an important role for LHRH in the regulation of the immune response [47].

Another major function of GH produced by cells of the IMMUNE SYSTEM is the induction of the synthesis of IGF-1, which, in turn, may inhibit the synthesis of both lymphocyte GH mRNA and protein. Our previous studies also show that both exogenous and endogenous GHRH can stimulate the synthesis of lymphocyte GH. Taken together, these findings support the existence of a complete regulatory loop within cells of the IMMUNE SYSTEM, and they provide a molecular basis whereby GHRH, GH, IGF-1 and their binding proteins may be intimately involved in regulating each other's synthesis. Furthermore, data obtained by immunofluorescence techniques suggest that the cells producing GH also produce IGF-1, which suggests that an intracrine regulatory circuit may be important in the synthesis of these hormones by cells of the IMMUNE SYSTEM [48]. Our findings in a T cell line show that endogenous GH promotes NO production, and up-regulation of IGF-1 RECEPTORS and Bcl2 protein along with an inhibition of superoxide formation, clearly establishing a role for lymphocyte GH in APOPTOSIS [49]. In follow-up studies in cells overexpressing GH, we also showed an increase in the expression of the IGF-2R RECEPTOR, transforming growth factor-β (TGF-β), and the inhibitor of differentiation-2 (ID-2) protein [50]. Overall, the data suggest that the regulation of lymphocyte growth by GH is complex, in that cells are stimulated to grow through events dependent on IGF-1, IGF-1R and ID-2, whereas growth appears to be limited by expression of IGF-2R and TGF-β1. A monocyte-derived cytokine, IL-12, has recently been shown to stimulate the synthesis of lymphocyte GH mRNA and the Th1 cytokine, IFN-γ [51]. The stress-activated hormones cortisol and CATECHOLAMINES decreased lymphocyte GH and the TH1 RESPONSE [51].

Interestingly, LYMPHOCYTES have also been suggested to be important sites of synthesis and action of ACETYLCHOLINE and CATECHOLAMINES since they contain both the enzymes necessary for biosynthesis of adrenaline (epinephrine) and ACETYLCHOLINE as well as the relevant RECEPTOR system [52, 53]. Recent work has identified a neural mechanism involving the vagus nerve and release of ACETYLCHOLINE that inhibits macrophage activation termed the "cholinergic anti-inflammatory pathway" [54]. The sensory afferent vague pathway may be activated by low doses of ENDOTOXIN, IL-1, or products from damaged tissues. The signal is relayed to the brain where activation of the efferent vague nerve releases ACETYLCHOLINE. ACETYLCHOLINE acts to inhibit macrophage release of the proinflammatory CYTOKINES TNF-α, IL-1, and IL-18, but not the anti-inflammatory cytokine IL-10. Thus, cholinergic neuron participation in the inhibition of acute INFLAMMATION constitutes a "hardwire" neural mechanism of modulation of the immune response.

Finally, calcitonin gene-related peptide (CGRP) has also been shown to be produced and secreted by human LYMPHOCYTES and may be involved in inhibition of T lymphocyte proliferation [55]. In another more recent study, substance P, the potent mediator of neuroimmune regulation, was shown to be up-regulated in LYMPHOCYTES by HIV INFECTION, implying it may be involved in immunopathogenesis of HIV INFECTION and AIDS [56]. NEUROPEPTIDES, by direct interaction with T cells, induce cytokine secretion, and break the commitment to a distinct T helper phenotype [57]. Thus, neurons are not the exclusive source of neurotransmitters and, therefore, provide

another instance of shared molecular signals and their RECEPTORS between the nervous and IMMUNE SYSTEM.

Functions of leukocyte-derived peptide hormones *in vivo*

Although much work needs to be done to fully establish the clinical relevance of leukocyte-derived neuroendocrine hormones, certain experimental models and clinical observations seem to support the view that leukocyte-derived hormones can also act on their classical neuroendocrine targets. The finding that cells of the IMMUNE SYSTEM are a source of secreted ACTH suggested that stimuli eliciting the leukocyte-derived hormone should not require a pituitary gland for an ACTH-mediated increase in corticosteroid. This seemed to be the case since Newcastle disease virus (NDV) infection of hypophysectomized mice caused a time-dependent increase in corticosterone production that was inhibitable by dexamethasone. Unless such mice were pretreated with dexamethasone, their spleens were seen to be positive for ACTH by immunofluorescence [58]. It has been estimated that if, *in vivo*, all the LEUKOCYTES produced ACTH at the levels seen *in vitro*, the contribution would be approximately 10% of the pituitary [30]. It, therefore, appears that the lymphocyte source of ACTH may mediate autocrine and/or paracrine effects, whereas endocrine effects, though possible, would be small. A more recent study has suggested that B LYMPHOCYTES can be responsible for extrapituitary ACTH production. In this report, hypophysectomized chickens were shown to produce ACTH and corticosterone in response to *Brucella abortus*, and the corticosterone response was ablated if B LYMPHOCYTES were deleted by bursectomy prior to hypophysectomy [59]. In children who were pituitary ACTH-deficient and pyrogen tested, an increase in the percentage of ACTH-positive peripheral blood LEUKOCYTES (PBL) was observed. Both the response in hypophysectomized mice and hypopituitary children peaked at approximately 6–8 h after administration of virus and typhoid vaccine, respectively [60]. Such studies have been furthered by a report that CRH administration to pituitary ACTH-deficient individuals results in both an ACTH and cortisol response [61]. In the cow, both transport stress [62] and pregnancy [63] stimulate ACTH secretion from peripheral LYMPHOCYTES, implying a role for lymphocyte ACTH in stress and probably recognition. Although more work needs to be done, it seems highly likely that locally produced MSH, *via* cells of the IMMUNE SYSTEM, may affect mast cell function and modulate immediate-type airway HYPERSENSITIVITY and INFLAMMATION [24]. The inhibitory effect of MSH on allergic airway INFLAMMATION appears to be mediated *via* enhanced IL-10 production since no effect was observed in IL-10 knockout animals [24]. Finally, LYMPHOCYTES from patients with active SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) produce increased amounts of PRL [64]. The extrapituitary PRL may initiate or maintain an aberrant immune process in SLE in IL-2-producing LYMPHOCYTES or disturb normal communication between the neuroendocrine system and the IMMUNE SYSTEM in SLE.

Gram-negative bacterial infections and ENDOTOXIN SHOCK represent another situation in which leukocyte hormones act on the neuroendocrine system. For example, ENDORPHINS have been implicated in the pathophysiology associated with these maladies since the opiate antagonist, naloxone, was shown to improve survival rates and inhibited a number of cardiopulmonary changes associated with these conditions [65]. Further, two separate pools of ENDORPHINS have been observed following bacterial LIPOPOLYSACCHARIDE (LPS) administration, and it was suggested that one pool might originate in the IMMUNE SYSTEM. Consistent with this idea is the observation that lymphocyte depletion, like naloxone treatment, blocked a number of ENDOTOXIN-induced cardiopulmonary changes. Our interpretation of these data is that lymphocyte depletion removed the source of the ENDORPHINS, while naloxone blocks their effector function. In a different approach, LPS-resistant inbred mice, which have essentially no pathophysiological response to LPS, were shown to have a defect in leukocyte processing of POMC to ENDORPHINS. If leukocyte-derived ENDORPHINS were administered to the LPS-resistant mice, they showed much of the pathophysiology associated with LPS administration to sensitive mice [66].

A role for the κ-opioid RECEPTOR in immunity has been studied in κ-opioid RECEPTOR knockout mice [67]. In these animals, there was an increase in splenocyte number and the humoral response, whereas no changes were observed in β-opioid RECEPTOR and δ-opioid RECEPTOR knockout animals. The data suggest that activation of κ-opioid RECEPTORS may exert a tonic inhibition of the Ab response. A similar finding was observed in β-endorphin-deficient mice [68].

The idea that non-immune tissues may harbor neuroendocrine hormone RECEPTORS, produce CYTOKINES, and indirectly influence immunity has also recently been suggested. The tissue-specific expression of IL-18 in the rat adrenal cortex, following ACTH treatment, but not in the spleen shows that adrenal cells may be the source of IL-18 during stress and not cells of the IMMUNE SYSTEM as expected [69]. Numerous *in vivo* experiments and studies in human disease have shown significant effects and/or changes of α-MSH [25]. Thus, chemokine, TNF-α and ADHESION MOLECULE production are reduced in animal models by systemic treatment with α-MSH, while in humans α-MSH plays an important role in fever and the acute-phase response. In the case of lymphocyte GH and IGF, there are now several reports showing the age-related differential expression of GH and IGF molecules and their RECEPTORS between LYMPHOCYTES from healthy adults, children, and newborns [70]. In addition, a role for lymphocyte GH *in vivo* has been suggested in HEMATOPOIESIS during fetal development and in the appearance of childhood acute lymphoblastic leukemia.

Another exciting new development in the opioid field has come with the demonstration that activation of endogenous opioids in rats through a cold water swim results in a local anti-nociceptive effect in inflamed peripheral tissue. This local ANTINOCICEPTION in the inflamed tissue apparently results from production by immune cells of endogenous opioids, which interact with opioid RECEPTORS on peripheral sensory nerves [71]. The findings identify locally expressed CRH as the main agent to induce opioid release within inflamed tissue. The opioid RECEPTOR-specific ANTINOCICEPTION in inflamed paws of rats could be blocked by intraplantar α-helical CRH or antiserum to CRH or CRH-antisense ODN. This latter treatment reduced the amount of CRH extracted from inflamed paws, as well as the number of CRH-immunostained cells [72]. An up-regulation of β-opioid RECEPTORS on sensory nerves and the accumulation of activated/memory T cells containing β-endorphin in inflamed tissue are consistent with the production of analgesia [73]. A more recent work suggests that the molecular pathway mediating the proinflammatory effects of peripheral CRH is through the induction of NF-κB DNA-binding activity [74]. A very recent work shows that LEUKOCYTES containing opioids are also important mediators of pain in a mouse model of neuropathy [75]. About 40% of immune cells that accumulate at injured nerves express opioid peptides that can be stimulated by CRH. The findings suggest that immune cell-derived opioids are not restricted to somatic pain, but are also critical for alleviation of pain from injury of nerves. It should also be noted that locally produced neuropeptide Y and its RECEPTOR are also involved in the pro-inflammatory responses of paw edema in the rat. In another model, studies employing a GHRH antagonist and GHRH RECEPTOR-deficient mice, the data suggest that GHRH plays a crucial role in the development of EAE [37]. Taken together, these observations offer new understanding and suggest new approaches for ameliorating pain and disease in normal, diseased and immunocompromised patients [76].

Regulation of nervous system by immune factors

Under pathophysiological conditions, inflammatory challenges at mucosal or cutaneous sites can modify properties of the PERIPHERAL NERVOUS SYSTEM (PNS) and the CNS. INFLAMMATION is associated with alterations of both neuronal electrophysiological behavior and neurochemical composition. Neurogenic INFLAMMATION is a local inflammatory reaction in response to infection, toxins or trauma mediated by NEUROPEPTIDES released from unmyelinated afferent nerve endings, and is characterized by vasodilatation, plasma extravasation and smooth muscle contraction [77]. NEUROPEPTIDES have been shown to modulate different aspects of mucosal function, and they play a

Table 5. Immunological factors involved in regulation of nervous system in chronic inflammatory diseases

Cytokines
Chemokines
Toll-like receptors
Immunoglobulin

role in the recruitment of granulocytes and lymphocytes, and in the modulation of mast cell activation [78–80]. Moreover, proliferation and growth of nerve fibers can be detected during inflammation at the site of the inflammatory response.

Indeed, directional interactions from neurons towards immune cells have been well described [81] and this mutual communication is present at mucosal and cutaneous sites between the immune system and PNS, vagal nerve fibers and spinal sensory nerves [82]. There is anatomical evidence demonstrating that mucosal lymphoid tissue is extensively innervated by noradrenergic and nonadrenergic non-cholinergic (NANC) neuronal pathways [83]. Below, the effects of immunological factors, from the innate and the adaptive immune system, on peripheral nerves at mucosal sites and the CNS, and its potential role in the development of chronic inflammatory diseases are reviewed (Tab. 5).

Cytokine-mediated neuronal activation

Cytokines are intracellular signaling proteins mainly produced by innate and adaptive immune cells. Their primary function is to regulate and coordinate the immune response. Several lines of evidence support the concept that cytokines are important mediators of chronic inflammation such as asthma, chronic obstructive pulmonary disease (COPD), multiple sclerosis and inflammatory bowel diseases. A broad range of cytokines have been implicated in the pathogenesis of inflammatory diseases, including the Th1 and Th17 pro-inflammatory cytokines (such as IL-1β, 2, 6, 12, 17, 18, 23, IFN-γ and TNF-α), Th2 cytokines (such as IL-4 and IL-13), and the anti-inflammatory cytokine IL-10.

The expression of cytokines and their receptors has been demonstrated on peripheral and central nerves. Several lines of evidence suggest indirectly that cytokines and their receptors are initiators of changes in the activity of NANC nerves associated with inflammation. Primary afferent fibers originate from NANC neurons in dorsal root ganglia (DRGs) and innervate peripheral mucosal and cutaneous sites or terminate in the dorsal horn of the spinal cord. Furthermore, a role for glial cells, macrophage-like cells that form part of the supporting structure of nervous tissue, in the maintenance and amplification of inflammatory disease has been described [84]. The majority of studies examining the neuronal effects of cytokines have been performed *in vitro* on DRGs that exhibit NANC functions.

One of the major cytokines involved in inflammatory diseases is TNF-α. In particular, the great clinical efficacy of the chimeric TNF-α Ab, infliximab, demonstrates a central role for TNF-α in inflammatory bowel disease and rheumatoid arthritis [85, 86]. In peripheral nerve injury, TNF-α expression is enhanced and can be transported axonally to DRG neurons and the spinal cord dorsal horn where it correlates with the expression of TNF-α R1 and R2 (p55 and p75) receptors that do not exist in DRG neurons usually [87–89]. More recently, it was demonstrated that inflammation-induced up-regulation of TNFR1 receptor on DRG neurons was shown to be linked to an increased expression of TNFR2 receptor on non-neural macrophage-like cells, probably glial cells [90]. The up-regulation of TNFR1 receptor on DRG neurons results in enhanced primary NANC nociceptive signaling, leading to increased firing rate of neurons [91–93]. TNFR2 receptor is possibly involved in the attraction of macrophages to DRGs [90].

In addition, it has been shown that TNF-α dose-dependently induces substance P release [94]. In visceral pain observed in inflammatory bowel disease and irritable bowel syndrome, substance P and TNF-α might be involved, causing spontaneous electrophysiological activity in NANC fibers [94, 95]. Based on immunocytochemical findings, NANC neurons from the DRG have been suggested to contain

TNF-α, possibly by synthesis or uptake from extraneuronal sources [96]. Recently, Aoki et al. [97] have shown that TNF-α induces axonal growth of nerve growth factor-sensitive DRG neurons. In contrast, others have provided evidence that neutrophil-driven neuronal cell death is dependent on TNF-α [98] *via* REACTIVE OXYGEN SPECIES.

IL-1β is also released during chronic inflammatory conditions. In the inflamed PNS, IL-1β indirectly induces increased SENSITIVITY of neurons to BRADYKININ, by inducing glial cells to release PROSTAGLANDIN E2. IL-1β induces the production of toxic mediators that will lead to neurodegeneration. Several reports have demonstrated that IL-1β can activate specific populations of enteric nervous system neurons [99, 100]. In the myenteric plexus, IL-1β specifically act on inhibitory motor neurons or interneurons expressing NO synthase, which can probably lead to the altered motor activity observed in inflammatory bowel diseases. In addition, IL-1β acts on vasoactive intestinal peptide (VIP)-positive NANC neurons that might be involved in the maintenance of local INFLAMMATION, CNS-mediated disease symptoms and development of neuronal HYPERSENSITIVITY associated with chronic inflammatory conditions.

On the other hand, several beneficial effects of IL-1β on the nervous system have also been reported, such as promotion of neuron survival partly *via* the synthesis of nerve growth factor [101]. INFLAMMATION-induced peripheral nerve injury results in the release of myelin-derived proteins, such as myelin-associated glycoprotein (MAG), which in turn inhibits neurite outgrowth. Recently, it has been shown that IL-1β prevents the effect of MAG on neurite outgrowth of DRGs.

In conclusion, enhanced release of TNF-α and IL-1β in inflammatory diseases by MAST CELLS, MACROPHAGES, glial cells and LYMPHOCYTES in close proximity of primary afferent NANC nerves in the mucosa or skin could be major player in the development of INFLAMMATION, dysfunction, pain and CNS-mediated disease symptoms such as fever, anorexia and depression.

Chemokine-mediated neuronal activation

A special group of CYTOKINES are the CHEMOKINES, originally described as chemotactic CYTOKINES involved in leukocyte trafficking. Chemokines are currently classified into four supergene families based on cysteine residues: CXC, CC, C and CX3C CHEMOKINES (see chapter A5). In general, CXC CHEMOKINES containing the Glu-Leu-Arg (ELR) motif (ELR$^+$CXC CHEMOKINES) are attractants for NEUTROPHILS and T LYMPHOCYTES; and ELR-CXC CHEMOKINES for B and T LYMPHOCYTES. CC CHEMOKINES induce CHEMOTAXIS of multiple subsets of LEUKOCYTES, such as MONOCYTES, BASOPHILS, DENDRITIC CELLS, MACROPHAGES, NK cells and T cells. C CHEMOKINES are important for trafficking of T cells to the thymus and the CX3C chemokine, fractalkine, also acting as an ADHESION MOLECULE, seems to be important for the infiltration of T cells, NK cells and MONOCYTES.

Research has shown that chemokine RECEPTORS are not restricted to LEUKOCYTES and are also found on neurons. Chemokine RECEPTOR activity has been detected on DRG neurons. Moreover, human PNS neurons and glial cells have recently been demonstrated to produce CHEMOKINES, such CXCL8 and CCL4, during intestinal INFLAMMATION [102, 103]. In addition, *in vitro* studies on co-cultures of human NANC neurons and epithelial cells show that TNF-α-induced epithelium results in an up-regulation of neuronal CXCL8 and CCL3 mRNA, mediated by the IL-1β RECEPTOR. Furthermore, pretreatment of neuronal cells with IL-1β resulted in CHEMOTAXIS of PBMC, which was CXCL8 dependent. Production of neuronal CHEMOKINES could therefore be responsible for the presence of LEUKOCYTES, MONOCYTES and LYMPHOCYTES in the PNS, which could ultimately lead to the development of neuropathies and CNS-mediated symptoms as observed in inflammatory diseases.

Chemokines interacting with their RECEPTORS on neurons can also alter the excitability of NANC nerves during INFLAMMATION. Up-regulation of CCL2 and its RECEPTOR CCR2 has been described in DRG neurons during injury [104, 105]. CCL2/CCR2 signaling increases the excitability of NANC nerves [106]. This suggests that pain during INFLAMMATION is exacerbated by pro-inflammatory CHEMOKINES. Immunohistochemistry and RT-PCR have revealed the expression of CCR1, CCR4, CCR5, CXCR4 and CX3CR RECEPTORS on populations of DRG that express substance P and the transient RECEPTOR potential vanilloid 1 (TRPV1) [107, 108]. Recently, it has been demonstrated that CCL3, by interacting with the

CCR1 RECEPTOR on DRG neurons, sensitizes transient RECEPTOR potential cation channel, subfamily V1 member 1 (TRPV1) RECEPTOR to become more susceptible to capsaicin [107]. A similar role for the interaction of CXCR4 and CXCL12 on DRGs in HIV-induced pain HYPERSENSITIVITY has been reported [109]. CCR1 RECEPTOR co-localized with the anti-pain, μ-opioid RECEPTORS on DRGs and pretreatment with CCL3, as well as CCL2, CCL5 and CXCL8, inhibited μ-opioid RECEPTOR-induced Ca^{2+} responses in neurons [110]. Chemokines may directly activate NANC nerves in INFLAMMATION. Interaction of CCL2 or CXCL1 with their respective RECEPTORS evoked intracellular Ca^{2+} elevations associated with the release of the neuropeptide CGRP from DRG neurons. Additionally, intraplantar injection of CCL2 and CXCL1 produced hyperalgesia in rats [111].

In conclusion, the mentioned studies suggest that pro-inflammatory CHEMOKINES can either sensitize or desensitize RECEPTOR functions on, or directly activate, peripheral NANC nerves. This cross (de)SENSITIZATION between neuronal (anti)pain and chemokine RECEPTORS or direct activation of NANC nerves may contribute to the development of pain observed in inflammatory diseases.

Toll-like receptor-mediated neuronal activation

TOLL-LIKE RECEPTORS (TLRs) are a relatively novel, but evolutionary old protein family. TLRs are the sentinels of the INNATE IMMUNE SYSTEM and play a crucial role in host defense against infection (see chapters A6 and A8). Every TLR recognizes one or more pathogen-associated molecular patterns of bacteria, viruses and fungi. Cell membrane-bound TLRs consist of TLR1, 2, 4, 5 and 6, whereas endosome-located RECEPTORS are represented by TLR3, 7, 8 and 9. TLR2, either alone or in association with TLR1 or TLR6, recognizes cell wall components of bacteria and fungi. TLR4 is highly specific for LPS, and TLR5 is specific for flagellin, a key organelle of motile bacteria. TLR3 is specific for double-stranded RNA, whereas TLR7 and 8 are specific for single-stranded RNA of different viruses. TLR9 recognizes DNA oligonucleotides containing CpG motifs from bacteria.

The role of TLR in interactions between the IMMUNE SYSTEM and nerves in inflammatory diseases is largely unexplored. TLRs have been reported to be expressed on neurons. However, a limited number of studies report on their function in INFLAMMATION. Recently, MICROARRAY analysis of mRNA for TLR1–9 on neuronal cultures revealed a low expression of TLR1, 3, 6, 7 and 8, intermediate levels of TLR2 and 4, and higher levels of TLR5 and 9 [112]. Primary murine cortical neurons exposed to IFN-γ express increased levels of TLR2, 3 and 4, and TLR2 and TLR4 play a role in pro-apoptotic pathways in neurons upon injury [112]. TLR4 was also found on enteric NANC nerves from the rat. Exposure of LPS to enteric NANC nerves resulted in neuronal cell death that was reduced by VIP release [113]. A role in neuronal APOPTOSIS and suppression of neurite outgrowth has also been reported for TLR8, which is expressed on neurons and axons [114]. In addition, TLR3 has been demonstrated on mouse peripheral nerves and in growth cones of neurons. TLR3-LIGAND stimulation resulted in a reduced neurite outgrowth [115]. Human and rat NANC nerves co-express TLR4/CD14 and the capsaicin RECEPTOR, TRPV1, indicating that pain due to INFLAMMATION may result in part from direct activation of NANC nerves by bacterial products such as LPS [116]. The expression of TLR4 in rat nodose ganglia might provide evidence for a novel pathway for LPS-induced afferent vagus nerve activation [117]. TLR3 seems to be involved in the production of CYTOKINES (TNF-α and IL-6) and CHEMOKINES (CCL5 and CXCL10) by human neurons [118, 119].

In conclusion, several inflammatory disease-associated TLRs, found on neurons, could exert their effects on the development of inflammatory bowel disease at different levels:

1. Inflammation-induced neuronal apoptosis and cell death
2. Sensitization of pain receptors and direct activation of NANC nerves inducing pain
3. Neuronal production of inflammatory disease-associated cytokines and chemokines, leading to infiltration and activation of leukocytes in the direction of nerves.

Immunoglobulin-mediated neuronal activation

IMMUNOGLOBULINS are synthesized by B LYMPHOCYTES and function as potent effector molecules (ANTIBODIES) in the humoral adaptive immune response (see chapters A3 and C2). Expression of immunoglobulin Fc RECEPTORS on neuronal tissue has been described in relation to neurodegenerative diseases, such as Parkinson's disease, which is suggested to be associated with increased activity of adaptive humoral immunity. Especially Fcγ RECEPTORS, the IgG RECEPTORS on microglial cells, have been studied in regard to nerve destruction in the CNS [120, 121]. One report describes the important physiological role of the FcγRIIB RECEPTOR on Purkinje neurons in the development and responsiveness of the cerebellum [122]. In the periphery, allergic reactions at sites such as skin and mucosa are known to be evoked by antigen-IgE or -IgG complexes *via* mast cell activation. Several observations suggest a mast cell-independent mechanism involved in IgE- and IgG-mediated allergic neuronal responses and direct antigen-specific activation of primary afferents. The first study on direct effects of IgG and its FcγR RECEPTOR on neuronal activation was published in 2002. In patients with amyotrophic lateral sclerosis, the FcγR mediates IgG-induced IgG uptake, increase in intracellular Ca^{2+} and ACETYLCHOLINE release from motor axon terminals [123]. Andoh and Kuraishi [124, 125] were the first to demonstrate that both FcγRI and FcεRI RECEPTORS are present on cultured mouse DRG. Moreover, IgG-antigen complexes were shown to increase the concentration of intracellular Ca^{2+} ions and to induce the release of substance P from DRG neurons [124]. The effect of IgE-antigen complexes on the activation of primary afferents remains to be investigated. Very recently, our data demonstrate that immunoglobulin-free light chains and IgE are able to mediate antigen-specific responses (SENSITIZATION and activation) in cultured murine DRG [126].

In conclusion, the direct immunoglobulin-neuron link may reveal a novel potential pathway of antigen-specific neuronal activation in sensations such as pain and itch, but also in local INFLAMMATION in chronic inflammatory diseases. Interestingly, although these immunoneuro-interactions are largely unexplored, VIP- and substance P-immunoreactive nerve C-fibers have been demonstrated to make direct membrane-membrane contact with immunoglobulin-producing plasma cells in colonic mucosa of patients with Crohn's disease [127], implicating interplay between these two cells type and/or their products in disease pathology.

Summary

The interactions between behavioral, neural, neuroendocrine, and immunological processes have been an area of research interest now for over 80 years. However, only in the past 30 years has active research begun to identify the mechanisms by which these complex systems may interact. There is now substantial evidence to support the presence of specific hormone RECEPTORS (i.e. neuroendocrine) and the ability of the cognate hormones to modulate the function of cells of the IMMUNE SYSTEM. This has been further supported by the demonstration of the innervation of LYMPHOID ORGANS. NANC nerves are critically involved in many pathways of the exacerbated innate and adaptive immune responses during inflammatory diseases at mucosal and cutaneous sites. In addition, there is overwhelming evidence that cells of the IMMUNE SYSTEM also produce neuroendocrine hormones. In some respects, the production and regulation of these peptides by LEUKOCYTES is remarkably similar to that observed in neuroendocrine cells. There are, however, a number of noteworthy differences, which suggest that rules that apply to pituitary hormone production are not necessarily applicable to the IMMUNE SYSTEM. Once produced, these peptide hormones seem to function in at least two capacities. They are endogenous regulators of the IMMUNE SYSTEM as well as conveyors of information from the immune to the neuroendocrine system. It is our opinion that the transmission of these molecules along with CYTOKINES to the neuroendocrine system represents a sensory function for the IMMUNE SYSTEM through which LEUKOCYTES recognize stimuli that are not recognized by the CNS and PNS [15]. These stimuli have been termed non-cognitive and include bacteria, tumors, viruses, and ANTIGENS. The recognition of such non-cognitive stimuli by immunocytes

is then converted into information, in the form of peptide hormones, neurotransmitters and CYTOKINES, which is conveyed to the neuroendocrine system and a physiological change occurs. The reciprocal situation, in which the stimulation of the IMMUNE SYSTEM functions primarily *via* the production of neuroendocrine hormones, CYTOKINES, CHEMOKINES, IMMUNOGLOBULINS, and TLR-mediated neuronal activation, is also clearly documented and convincingly establishes the bidirectional nature of the communication between the immune and nervous systems. Therefore, it is abundantly clear that BIDIRECTIONAL COMMUNICATION between the immune and nervous systems is mediated by shared NEUROPEPTIDES and CYTOKINES and their respective RECEPTORS. A balance in these regulatory loops can be perceived in the normal organism as a physiological mechanism of achieving homeostasis and resistance to infection and disease. Further dissection of RECEPTOR-mediated and intracellular signal pathways in the neuron-immune interactions will help to develop more effective therapeutic approaches for chronic inflammatory diseases.

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B

Immunodiagnosis

Antibody detection

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Introduction

The ACCURATE and reliable determination of biologically active endogenous compounds in plasma, urine and other body fluids for scientific or diagnostic purposes has been a challenge for many decades. In the past, the assessment of such compounds was difficult and tedious because specific and practical analytical tools were not available. In the early days, decisive and convincing conclusions about the significance of a biologically active molecule in disease or health could only be made after purification and isolation of the ANALYTE and identification of its chemical structure. A definite improvement in the analysis of biologically active endogenous compounds was the introduction of bioassays using an intact animal model or *in vitro* tissue preparations. Although the bioassays possessed sufficient SENSITIVITY, there were problems with their lack of SPECIFICITY. Other analytical procedures such as liquid chromatography, electrophoresis or photometric procedures have also been developed for *in vitro* diagnosis. However, these approaches are either tedious or time-consuming and require expensive equipment and specially trained personal. A landmark in diagnostics was the introduction of immunoassays (IAs), which are inexpensive and easy to perform with high reproducibility, SENSITIVITY and SPECIFICITY.

Basic principle of immunoassays

IAs are based on an antigen-ANTIBODY (Ab) reaction utilizing the exceptional capability of the IMMUNE SYSTEM to produce specific Abs that can recognize and discriminate between a practically infinite number of foreign compounds. The basic requirement for setting up an IA is the production of a specific Ab to a given antigen or hapten by immunizing animals such as mice, rabbits, goats or horses. High molecular weight compounds such as proteins are immunogenic and may serve as ANTIGENS, which can be injected into the host animal directly to produce Abs. In contrast, low molecular weight compounds such as drugs, amines, peptides or steroids are HAPTENS, which do not induce immune responses. They can be rendered immunogenic by coupling to a CARRIER. The Abs obtained from the serum after several booster injections with the immunogen are HETEROGENEOUS polyclonal Abs (pAbs). A more sophisticated approach was the production of monoclonal Abs (mAbs), which was first introduced by Köhler and Milstein in 1975 [1]. This technique allows the production of HOMOGENEOUS, single Abs in nearly infinite quantities with high SPECIFICITY for a certain antigen. The method involves the isolation of spleen cells from an immunized animal containing Ab-producing B cells. The B cells are then fused with myeloma cells. After selection and screening of the desired Ab-secreting cell line (hybridoma), the Ab can be harvested in the supernatant. These hybridomas can be grown in large volumes for the production of huge quantities of the mAb.

The essentials for the characterization of the Abs produced are high AFFINITY, SPECIFICITY and SENSITIVITY. The AFFINITY is a measure of the strength of the binding interaction between the antigen and the Ab and can be experimentally determined by the dissociation constant (*K*d) of the antigen to the Ab. The lower the *K*d the greater the AFFINITY. The SPECIFICITY refers to the specific recognition of the ANALYTE by the Ab without cross-reacting with closely related or structurally similar ANALYTES. This in turn is closely related to the ability of the Ab to discriminate between negative

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and positive samples. The SENSITIVITY describes the detection limit and is defined by the dose-response curve of the antigen to the Ab. The lower the detection limit, the higher the SENSITIVITY.

Antibody structure

Abs, among other serum proteins such as albumins, represent the gammaglobulin or immunoglobulin (Ig) fraction, based on their electrophoretic mobility. They are glycoproteins which are chemically very similar in structure and are constructed from two identical light chains of approximately 50 kDa and two identical heavy-chains of approximately 110 kDa. Each of the two light chains are attached to one heavy chain *via* disulphide bonds. Likewise, the heavy chains are bound to each other by disulphide bridging. Two locally distinct binding domains are prominent on Ig molecules. One is the antigen-binding site (Fab) for the binding and recognition of ANTIGENS and the other is the RECEPTOR-binding site (Fc) for binding to specific RECEPTORS on various cells involved in immunological functions such as mononuclear PHAGOCYTES, NATURAL KILLER CELLS, MAST CELLS or basophilic LEUKOCYTES (Fig. 1). Although Igs share an overall similarity, they can be divided into different classes and subclasses according to their size, charge, solubility and their behavior as ANTIGENS. At present, the classes of Ab molecules in humans can be divided into IgA, IgD, IgE, IgG and IgM. IgA and IgG can be further subdivided into their subclasses IgA1 and IgA2 and IgG1, IgG2, IgG3 and IgG4 (see chapters A3 and C2).

Clinical relevance of antibody detection

Initially, the identification and characterization of specific Abs associated with pathophysiological conditions was generally constrained to scientific purposes. However, in many instances the measurement of specific Abs evolved into clinically relevant diagnostic markers in health and disease. Medical

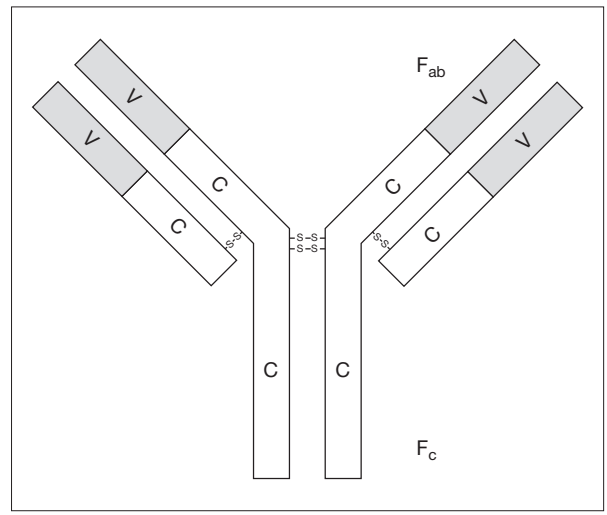

FIGURE 1. STRUCTURE OF IMMUNOGLOBULINS
For further details see text.

conditions in which the determination of specific Abs is used routinely are bacterial and viral infections, infestation with parasites, AUTOIMMUNE DISEASES and allergies.

Microbial infections

The determination of Abs in infectious diseases has been known and used for a long time. Bacterial-specific Abs of the IgG and IgM class are routinely detected in the serum of patients infected with different bacteria such as *Borrelia burgdorferi* (Lyme disease), *Chlamydia trachomatis* (sexually transmitted infection of the urogenital tract), *Legionella pneumophila* (Legionnaires' disease), *Staphylococcus* and *Streptococcus*, and *Treponema pallidum* (lues/syphilis).

The assessment of IgG and IgM Abs is also a very valuable parameter in the diagnosis of viral infections, such as hepatitis, measles, epidemic parotitis and rabies, and infections with the Epstein-Barr virus (mononucleosis), herpes virus (herpes simplex and herpes zoster) and arbor virus (tick encephalitis).

Infestations with parasites leading to diseases such as leishmaniasis, amoebiaisis, malaria, toxoplas-

mosis, schistosomiasis, echinococcosis, tichinellosis, filariasis or others can induce the formation of Ig molecules of different classes. In the diagnosis of parasitosis it is recommended that the identity of the parasite be ascertained first. In addition, serological methods are available to identify circulating ANTIGENS, antigen-Ab complexes or circulating Abs.

Autoimmune diseases

Another area of pathophysiological abnormalities in which the measurement of Abs has predictive and diagnostic value is that of the numerous AUTOIMMUNE DISEASES in which self-TOLERANCE of the IMMUNE SYSTEM against its own ANTIGENS is abrogated. Examples of an organ-specific AUTOIMMUNE DISEASE are Hashimoto's thyroiditis with circulating Abs against thyroglobulin, and myasthenia gravis with auto-Abs against the ACETYLCHOLINE RECEPTOR. Examples of non-organ-specific AUTOIMMUNE DISEASES are Sjögren's syndrome, RHEUMATOID ARTHRITIS, scleroderma and SYSTEMIC LUPUS ERYTHEMATOSUS, affecting the skin, joints and muscles with Abs against nuclear ANTIGENS such as DNA, RNA or histones.

Allergy

The most important Igs for the *in vitro* diagnosis of allergic diseases, either immediate-type HYPERSENSITIVITY reactions (e.g., rhinitis, conjunctivitis, allergic bronchial ASTHMA and ANAPHYLAXIS) or LATE PHASE reactions (e.g., allergic contact dermatitis), are Abs of the IgE class [2, 3]. Clinically relevant measurements include that of total IgE or allergen-specific IgE in the patients' serum for the determination of IgE-mediated SENSITIZATION. Total serum IgE levels of >100 kilo units (kU)/L in adults and children are a good indicator for atopy, a disease characterized by familial HYPERSENSITIVITY to exogenous environmental agents associated with high IgE Ab titers and altered reactivity against various pharmacological stimuli. However, high total IgE Abs can also be induced by parasitic worm infestations. Extremely high values, higher than 10 000 kU/L, are indicative of IgE-producing myelomas. The measurement of allergen-specific IgG Abs, as IgE-blocking Abs, to monitor the success of immune therapy with insect venoms in patients with hymenoptera venom ALLERGY has been used tentatively, but with discrepant results.

Antibody-detection methods

The detection of Abs in the circulation or in tissue has become a useful analytical tool for the *in vitro* immunodiagnosis of numerous diseases. Several immunological techniques are available for the routine identification of IgA, IgD, IgE, IgG and IgM Ab classes in the clinical chemistry laboratory. The most commonly used methods are discussed briefly.

Immunoprecipitation assay

Immunoprecipitation is a very simple and easy to perform *in vitro* assay for the identification and semi-quantitation of soluble Abs. The addition of the antigen to the Ab results in the formation of a three-dimensional, insoluble network of aggregates, which precipitate and can be detected with a nephelometer. The assay is very similar to a volumetric acid/base titration. The bulk of precipitate, formed at equivalent concentrations of Ab and antigen, is a measure for the concentration of the Ab. The assay can also be used in reverse to measure the antigen concentration by adding Abs.

A variation of the immunoprecipitation assay is the hemagglutination test and the complement fixation test. The hemagglutination test allows the identification of Abs to red blood cell ANTIGENS or the detection of Abs to ANTIGENS that are covalently or non-covalently attached to the red cell surface. The complement fixation test is a three-step assay in which the Ab-containing serum is initially incubated with a fixed amount of antigen to form an immune complex. In the second step, complement is added and is firmly incorporated by the immune complexes. Finally, red blood cells are added as indicator cells. Red blood cells will only be lysed if immune complexes have been generated.

Immunocytochemistry

Immunocytochemistry is a technique for the detection of an antigen/Ab *in situ* in tissue slices. Immediately after isolation tissues are fixed or frozen. Frozen tissue or tissue embedded in various embedding media is cut into thin slices and then immobilized on a slide. Frozen tissue is then fixed with formaldehyde, glutaraldehyde, alcohol or acetone. Slides are subsequently incubated with a specific primary Ab directed against the antigen to be detected. In the direct assay, the primary Ab is chemically coupled to a fluorescent dye (rhodamine, fluorescein), which allows the detection of the ANALYTE by fluorescence microscopy. In the indirect assay, excess primary Ab is thoroughly washed off, and the tissue is incubated with a secondary Ab to form a sandwich. The secondary Ab can be fluoresceinated or coupled to an enzyme, e.g., alkaline phosphatase (ALP) or peroxidase, which allows the visualization of the ANALYTE by fluorescence microscopy or by light microscopy after addition of a colorless SUBSTRATE that is enzymatically converted to a colored product. Only cells that contain the ANALYTE light up under the microscope.

Immunoblotting

The immunoblot or dot-blot technique is similar to the immunoprecipitation assay. However, in immunoblotting the antigen-Ab reaction takes place in the solid phase, whereas in the immunoprecipitation assay the Ab reacts with the antigen in solution. The assay utilizes the capability of nitrocellulose membranes to bind ANTIGENS. ANTIGENS are applied in small dots, and the membranes are dried. The membranes are treated with ovalbumin, gelatin or milk proteins to prevent non-specific adsorption. After blocking, the membranes are incubated with the serum and dilutions of the serum containing the Ab. The membranes are washed to remove excess Ab. The membranes are then incubated with a secondary Ab raised against the Ab of interest, which is conjugated with an enzyme. The formation of the antigen-Ab-secondary Ab complex can be visualized by adding a SUBSTRATE, which is converted by the enzyme attached to the secondary Ab to yield a colored or light-emitting (chemiluminescent) spot. The intensity of the spots is proportional to the amount of Ab present in the serum samples (Fig. 2, left panel). However, dot-blot results alone do not reveal whether the antigen is made up of one or several antigenic components.

Further characterization of the Ab can be achieved by separating the ANTIGENS electrophoretically. The separated components are transferred from the gel to a nitrocellulose membrane, a process called Western blotting. The membranes are then treated like dot blots as outlined above. This methodology combines the high resolving power of electrophoresis and the discriminating power of an immunological reaction. Components recognized by the Ab show up on the Western blot as colored bands (Fig. 2, middle and right panels).

Immunosorbent assays

Immunosorbent assay (IA) techniques are widely used for the measurement of serum IgE and IgG Abs. The concept of IAs is basically very similar to immunoblotting. However, the major difference between immunoblotting and IA is that the amount of Abs can be quantified. An antigen (e.g., an allergen extract from chicken meat, grass or tree pollen, or house dust mites) is attached to an inert matrix, such as the wall of a reaction vial or a microtiter plate well, or chemically coupled to a paper disc. In a first-step reaction, the serum of an allergic patient is incubated with the allergen-carrying matrix. IgE molecules that recognize the allergen are bound. After removal of excess serum, a secondary Ab, in this case an anti-human IgE Ab raised in rabbits, goats or horses, is added, which forms an allergen-IgE-anti-IgE Ab complex (second-step reaction). Excess of the secondary Ab is also removed by washing. The formation of the allergen-IgE-anti-IgE Ab complex depends on the amount of specific IgE present in the serum sample. Since the secondary Ab or detecting Ab carries a covalently coupled LABEL or tag, the formation of the allergen-IgE-anti-IgE Ab complex, a sandwich-like structure, can be monitored. Utilizing a standard curve with increasing concentrations of the ALLERGENS, the signal obtained with the complex in

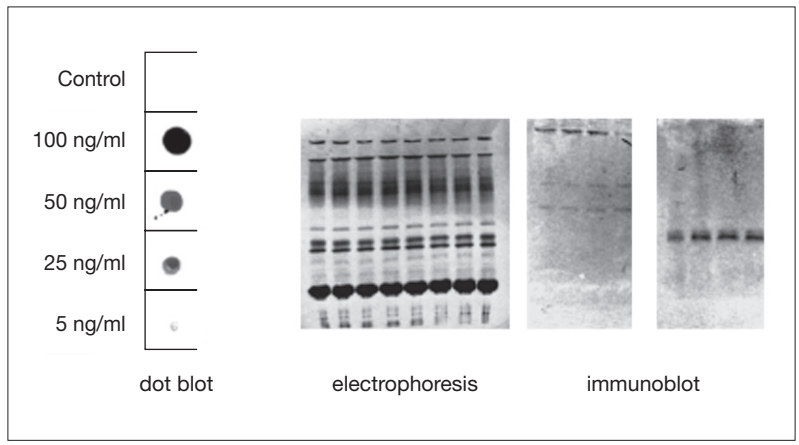

FIGURE 2. DOT BLOTS AND IMMUNOBLOTTING
Specific Abs to an antigen, e.g., an allergen extract, can be detected with the dot-blot technique. Further characterization of the Abs or the antigens present in the allergen extract can be achieved by immunoblotting. For further details see text. Lane 1, molecular weight protein markers; lanes 2–8, different allergen extracts.

the serum sample can be compared with the signal of the standard curve, permitting quantitation of the IgE Abs. The assay format of an IA in general is summarized in Figure 3.

The radio-allergo-sorbent test (RAST) is a radioimmunoassay (RIA) [4] in which the ALLERGENS are chemically coupled to a paper disc and the secondary Ab is radioactively labeled with ^{125}I. Similarly, the assay can be performed as an enzyme immunoassay (EIA) in which the secondary Ab is labeled with the enzyme β-galactosidase, which can react with a colorless SUBSTRATE to form a colored reaction product. In addition to the RAST or EIA, a RAST or EIA inhibition assay can be performed to confirm and validate the results [5]. The serum samples are first incubated *in vitro* with increasing concentrations of the ALLERGENS prior to the RAST or EIA. The binding inhibition of the Ab can be illustrated by a dose-response curve, which inversely correlates with the concentration of ALLERGENS added; low allergen concentrations still give a high signal in the RAST or EIA, whereas the signal vanishes with high concentrations. The concentration of the allergen at 50% inhibition (IC_{50}) can be calculated from the dose-response curve. A low IC_{50} is a good indicator for a high AFFINITY and SPECIFICITY of the Ab for the ALLERGENS (Fig. 4).

Automated systems to analyze specific IgE antibodies

Based on the RAST or EIA principle, different automated systems have been developed to detect allergen-specific IgEs. Three-dimensional cellulose sponges are used in the ImmunoCAP system (Phadia Uppsala, Sweden). The ALLERGENS are covalently coupled to the sponges (in the form of a small cap) and detection of allergen-specific IgE in the patient's serum takes place with an enzyme-coupled anti-IgE Ab. Other autoanalyzer platforms have been devel-

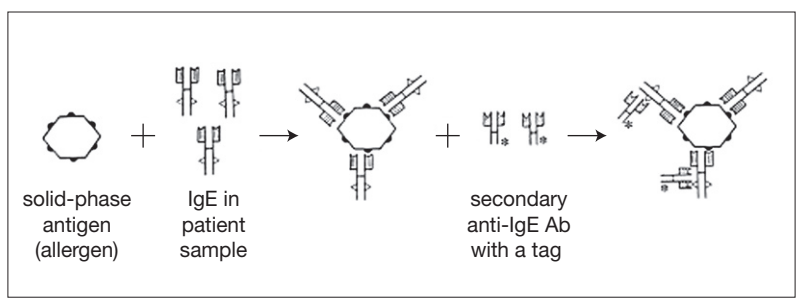

FIGURE 3. IMMUNOSORBENT ASSAY FORMAT
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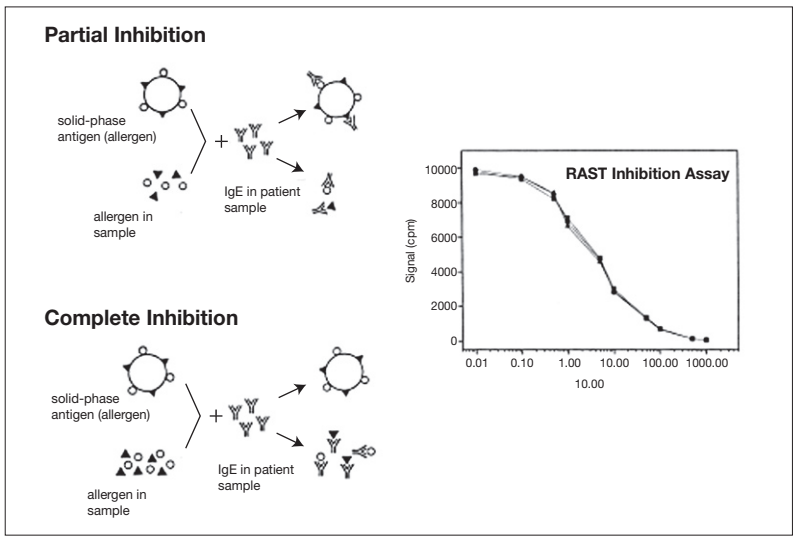

FIGURE 4. RAST INHIBITION ASSAY
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oped by Siemens (Immulite System) and Hycor-Agilent Technologies (Hytec-288 system). In the Immulite system, biotinylated ALLERGENS are used, which are captured on an avidin solid phase, while the Hytec system uses a cellulose wafer to which the allergen is covalently coupled. All of these methods have enhanced reproducibility, precision and SENSITIVITY and are automated to prevent human error. However, although all three systems are calibrated to World Health Organization (WHO) serum standards, intra-method differences with respect to quantitative estimates of allergen-specific IgE concentrations make it difficult to compare analyses performed on different systems [6].

Microarray systems

Several new MICROARRAY systems have emerged for the detection of specific Abs in allergic or AUTOIMMUNE DISEASES. Using a biochip, semi-quantitative detection of IgE binding to more than 100 proteins derived from over 40 common ALLERGENS can be achieved in only 20 µL serum or plasma [ImmunoCap Immuno solid-phase allergen chip (ISAC), Phadia, Sweden]. Bound IgE is detected with a fluorescently labeled anti-IgE Ab [7]. Protein arrays to profile auto-Abs have been used in research on MULTIPLE SCLEROSIS. Using this approach, 37 000 different expression CLONES from a human fetal brain cDNA library were spotted on poly(vinylidene fluoride) (PVDF) membranes. Membranes were incubated with individual cerebrospinal fluid samples to detect binding of auto-Abs [8].

Microsphere-based flow cytometric analysis is an important new technique used, for example, in clinical diagnosis [9]. The commercially available kits employing this technique allow simultaneous, quantitative detection of up to 100 different ANALYTES [Luminex xMAP, VeraCode/BeadXpress, Cytometric Bead Array (CBA) BD Biosciences], while instruments to run 500-plex testing in single samples have now also entered the market (FLEXMAP 3D®, Luminex). In these platforms, sets of Ab-coupled microspheres are used with different internal fluorescent labels (Fig. 5). When passing the laser beam through the analysis cuvette, multiple microspheres can be differentiated from each other. With the xMAP technology, two different fluorescent dyes (one red and one far-red) are incorporated at different concentrations into the microspheres and are analyzed in a specialized analyzer (Luminex, USA). With the VeraCode technology, different Ab-labeled microbeads are encoded by a holographic inscription, and analysis is performed on an Illumina reader. Analysis of CBA microbeads (BD Biosciences) can be per-

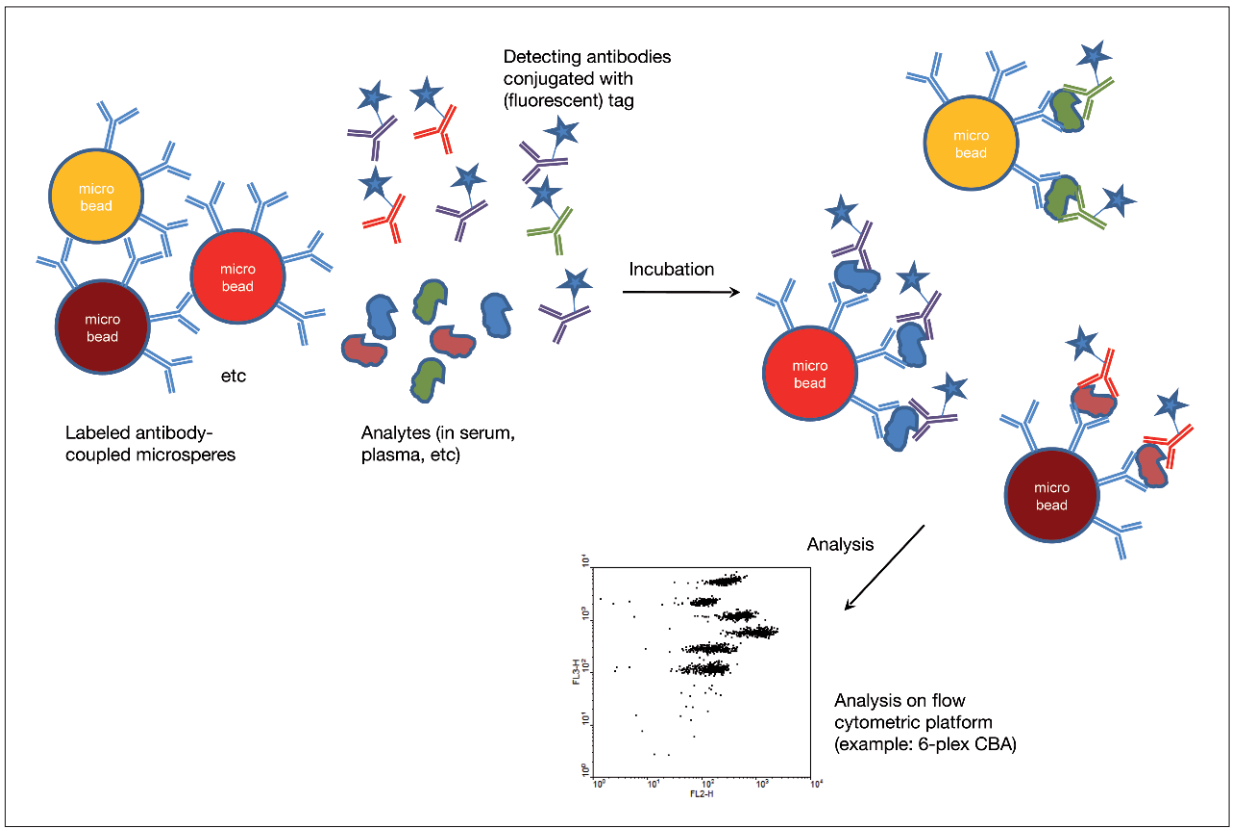

FIGURE 5. PRINCIPLE OF MICROSPHERE-BASED FLOW CYTOMETRIC ANALYSIS
Biological samples are incubated with a mixture of Ab-conjugated microbeads and tagged detecting antibodies. After incubation, samples are analyzed on a flow cytometric platform.

formed on regular flow cytometers. Beads are differentiated based on their incorporated fluorescent dye intensity in FL3 (fluorescent channel) and FORWARD SCATTER (FSC) and SIDE SCATTER (SSC). Binding to bead-coupled Ab is detected in channel FL2.

Summary

Ab detection is crucial for the differential diagnosis of many different pathological conditions. Determination of specific Abs to bacterial and viral pathogens as well as to parasites enables the correct therapeutic measures to be taken. Abs to organ-specific and systemic AUTOIMMUNE DISEASES are predictive for prognosis, and detection of IgE class Abs is essential for the diagnosis of immediate HYPERSENSITIVITY reactions.

Methods for detection of Abs include immunoprecipitation assay, in which antigen-Ab complex aggregates are detected, often by hemagglutination; immunocytochemistry, for *in situ* Ab detection in tissue slices; immunoblotting (dot-blot technique) whereby antigen-Ab aggregates are trapped on membranes and then detected with a secondary Ab to yield spots; and immunosorbent assays, which are similar to immunoblotting but, by using a tagged secondary Ab, the primary Ab can be quantified.

A variety of immunosorbent kits are available, which permit rapid, specific, ACCURATE and sensitive

detection, particularly of IgE Abs. Several automated systems permit clinical analysis of antigen-specific Abs in body fluids, while antigen microarrays can be used to determine hitherto undefined specifities of (auto)Abs. Ab microarrays, e.g., for cytokine analysis, are also very helpful to delineate the outcome of complex interactions of immune cells.

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Immunoassays

Jeffrey K. Horton and Michael O'Sullivan

Introduction

For approximately five decades, immunoassays have been the method of choice for quantifying low concentrations of ANALYTES in complex biological fluids. The procedure is equally applicable to the measurement of small molecules such as drugs and large molecules such as proteins. The technique combines SENSITIVITY and SPECIFICITY with ease of use.

Immunoassays are used in basic biological research to investigate the physiological and possible pathological role of a wide range of biologically active substances including cyclic nucleotides, PROSTAGLANDINS, LEUKOTRIENES, GROWTH FACTORS and CYTOKINES [1]. Such research often leads to the identification of new potential targets for therapeutic agents. The assays are also used in the pharmaceutical industry in many aspects of the drug development process. These range from drug screening, toxicological, pharmacological and pharmacokinetic studies through to clinical trials. Immunoassays have perhaps had their greatest impact in the area of clinical diagnostic tests. The technique has been employed for many years in hospital clinical biochemistry laboratories to diagnose disease and metabolic disorders. More recently, applications of this technique have moved out of these core areas into such diverse situations as the biotechnology industry, the food safety industry and even to "over the counter" applications such as home pregnancy and self-test devices. In fact it is difficult to think of any area of the biological sciences where immunoassays have not had a significant impact.

The technique was introduced in 1959 by Berson and Yalow [2]. The combination of a signal that can be easily detected and a protein molecule, which binds specifically and with high AFFINITY to the ANALYTE of interest, lies at the heart of all immunoassay procedures. Assay designs have proliferated over the last 40 years, as have the different types of signal reagents and detection systems. Sophisticated instruments with associated computer hardware have been developed with the aim of increasing sample throughput. This chapter discusses and highlight the main elements of the subject but cannot hope to be an in-depth review of the whole field. For the interested reader, "The Immunoassay Handbook, Third Edition" published in 2005, provides a comprehensive review of the area (see *Selected Readings*).

Basic principles of assay design

Competitive immunoassays

In the competitive immunoassay (also termed "labelled ANALYTE") approach there is competition between labelled and unlabelled ANALYTE for a limited amount of binding sites on an ANTIBODY. ANTIBODY-bound ANALYTE is separated from unbound ANALYTE and the proportion of LABEL in either fraction is analysed. A curve can then be plotted of the percentage of TRACER bound to the ANTIBODY against a range of known standard concentrations. The concentration of unknown ANALYTE present in the sample can then be determined by interpolation from the standard curve. The principle of the assay is illustrated in Figure 1. In this example there is competition between unlabelled and enzyme-labelled ANALYTE for the capture ANTIBODY on the solid phase. The amount of enzyme-labelled ANALYTE bound to the solid phase is inversely proportional to the concentration of unlabelled ANALYTE. Following a wash step, bound

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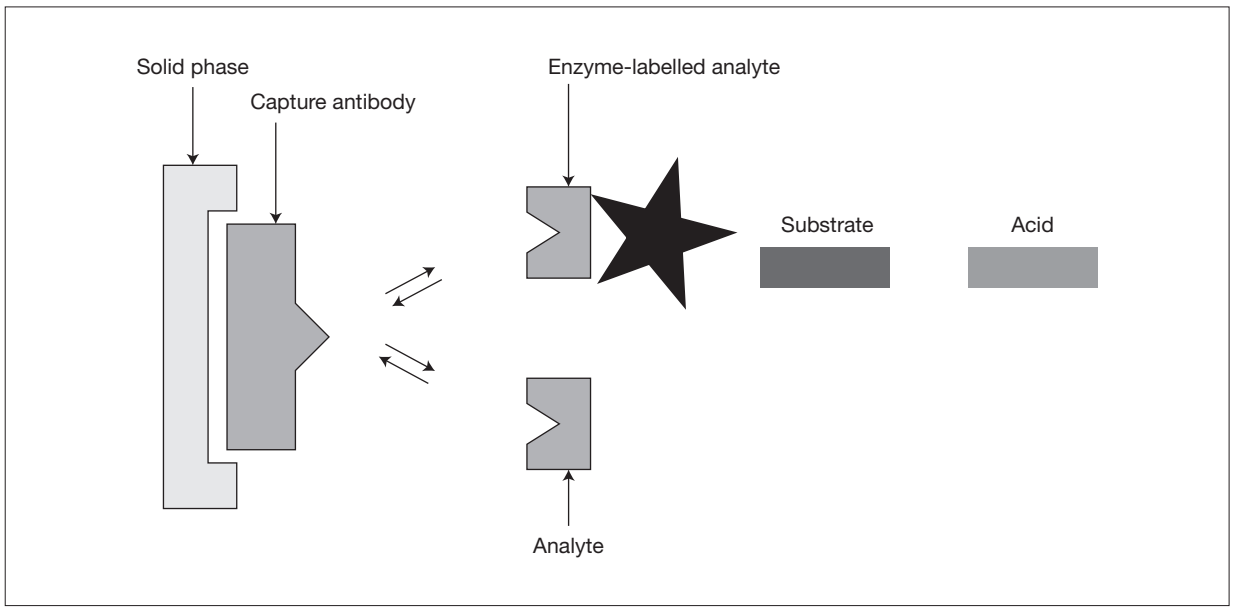

Figure 1. Principle of a competitive immunoassay

enzyme activity is determined by incubation with the SUBSTRATE. After a timed interval, the reaction is terminated with dilute acid and the resultant colour intensity determined in a spectrometer.

Although an ANTIBODY is usually used in these assays, there may be circumstances where it is more appropriate to use a naturally occurring binding protein or RECEPTOR [1]. This does not affect the principle of the assay. This assay format has the advantages that only one ANTIBODY is required and it uses relatively small amounts of the sometimes scarce ANTIBODY reagent. It has the disadvantages that assay SENSITIVITY is limited by ANTIBODY AFFINITY, the assays have a relatively narrow dynamic range and the labelling process may alter the binding characteristics of the labelled ANALYTE. This format tends to be favoured for small ANALYTES.

Immunometric assays

The immunometric (also termed "labelled ANTIBODY") approach differs from the competitive approach in a number of ways. In its most common format it involves two ANTIBODIES both of which are specific for the ANALYTE. One of these ANTIBODIES is labelled; the other is attached to a solid phase. The sample containing the ANALYTE is added and followed by the labelled second ANTIBODY Unbound LABEL is removed by washing. The amount of LABEL bound to the solid phase is related to the amount of ANALYTE in the sample. A standard curve can be constructed using known quantities of ANALYTE and the concentration of ANALYTE in the sample can be determined by interpolation from the curve. This approach is called a two-site immunometric assay. For obvious reasons this format is commonly referred to as a "sandwich assay". The principle of the assay is illustrated in Figure 2. The so-called detection ANTIBODY is labelled with an enzyme. Following washing to remove unbound LABEL, the bound enzyme activity is determined by incubation with its SUBSTRATE. The reaction is then terminated with dilute acid and the resultant colour intensity determined in a spectrometer.

This method has the advantages that it tends to be more sensitive and PRECISE than the competitive approach. It also tends to have a wider

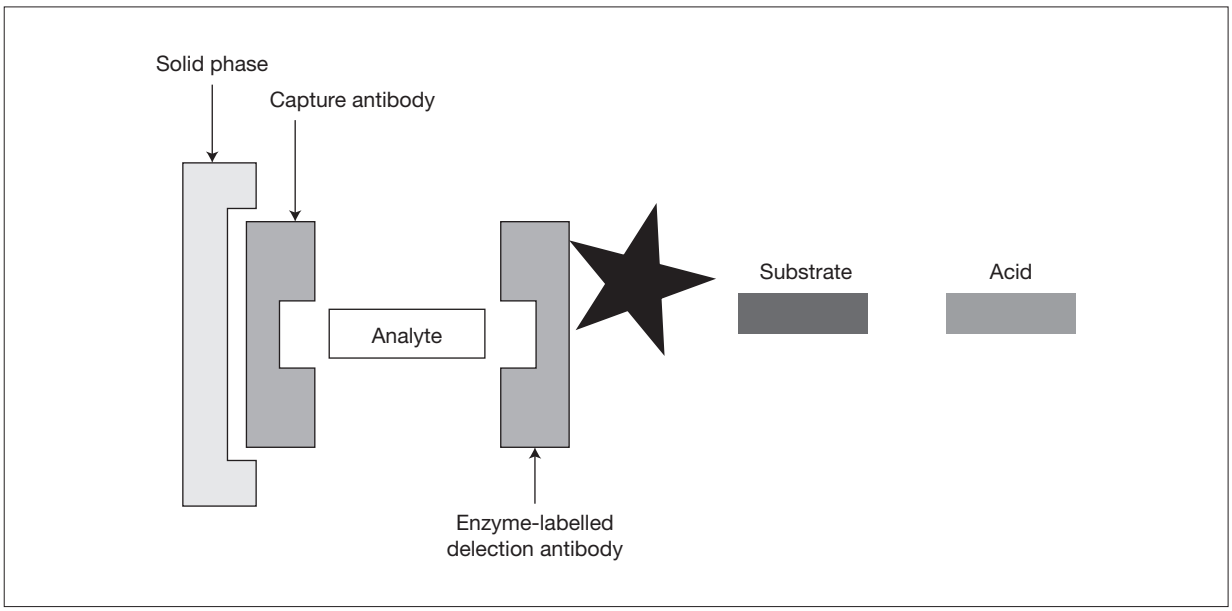

FIGURE 2. PRINCIPLE OF A TWO-SITE IMMUNOMETRIC SYSTEM

dynamic range and there is no requirement to LABEL the ANALYTE. The major disadvantages of the technique are the high consumption of ANTIBODY and the requirement for two ANTIBODIES. Immunometric assays are the favoured technique for quantifying large molecules. It cannot be applied to small molecules due to the size restraint on binding two large ANTIBODIES to one small molecule at the same time. These two basic approaches have been the subject of endless permutations, some of which are touched upon in later sections of this chapter.

Homogeneous assays

The assay formats described above suffer from one significant disadvantage in that they are HETEROGENEOUS ASSAYS, with a requirement to separate bound from free TRACER. This is a labour-intensive step that is difficult to automate and introduces significant imprecision into the assay. In an attempt to overcome this problem, considerable effort, ingenuity and money have been invested in developing HOMOGENEOUS ASSAYS, which do not require a separation step. Several successful methods have been developed for the quantification of small molecular weight ANALYTES, but the methods lack SENSITIVITY and are also not generally applicable to the measurement of large molecules. One exception is the technique termed "scintillation proximity assay, SPA", which is discussed later in the chapter.

Components of immunoassays

Tracers

Radioisotopes

For many years after the technique was introduced radioisotopes were used, virtually exclusively, as the assay TRACER. Radioactive iodine (^{125}I) was the favoured LABEL: its high specific activity providing good assay SENSITIVITY and a reasonably long half-life giving adequate reagent shelf life. It was also easy in many cases to prepare labelled proteins. The equipment required to measure radioactive decay was

also readily available. Finally, the rate and measurement of radioactive decay was not affected by the SAMPLE MATRIX. In most situations ^{125}I remained the LABEL of choice for the next 20 years and is still used even today.

Tritium tracers were also widely used for small molecule assays. Such tracers are readily commercially available and have a long shelf life. The tritiated molecule is also virtually identical to the non-labelled molecule. Many other labelling techniques change the structure of the labelled molecule, which often results in differences in AFFINITY of the interaction of the ANTIBODY with the ANALYTE and the LABEL. This can adversely affect assay performance. For these reasons immunoassays for small molecules can often be set up most quickly using tritium tracers. However, tritium tracers do have significant disadvantages. In particular, their relatively low specific activity demands long count times and their measurement requires the use of organic scintillant cocktails. For these reasons tritium-based assays have a tendency to be replaced by ^{125}I and non-isotopic tracers, if and when labelling problems are overcome.

Non-isotopic tracers

Radioisotopes are perceived as posing a potential health risk and there certainly are regulatory problems associated with their use and disposal. In addition, when non-isotopic assays were first being developed, some researchers believed that there would be advantages associated with the use of non-isotopic tracers, although this was hotly disputed at the time by some radioimmunoassay experts. In practice the development of satisfactory labelling techniques and suitable assay designs for non-isotopic tracers did prove difficult. A major breakthrough came with the introduction of 96-well microtitre plates with associated washing and measuring equipment [3].

Today enzymes are the most widely used tracers [4]. When used in combination with colourimetric end points they provide highly sensitive, robust, PRECISE, ACCURATE and convenient immunoassays [5]. Inexpensive automatic colourimetric multiwell plate readers are readily available. Many commercial kits are on the market, which enable relatively inexperienced workers to measure pg/mL levels of biologically active compounds in complex biological fluids with inexpensive, readily available laboratory equipment. Horseradish peroxidase in combination with a ready to use formulation of its SUBSTRATE 3,3'5,5'-tetramethly benzidine has proved extremely popular. Many other non-isotopic tracers have been used, of which fluorescent and luminescent labels have stood the test of time.

In some assays the detection ANTIBODY is labelled with biotin rather than an enzyme. The biotinylated ANTIBODY is used in combination with a streptavidin/horseradish peroxidase conjugate. Streptavidin has a very high AFFINITY for, and binds very quickly to biotin, so linking the biotinylated ANTIBODY non-covalently to the enzyme. This approach tends to LABEL the ANTIBODY more consistently and to give a modest two-to four-fold increase in assay SENSITIVITY. A greater increase in SENSITIVITY can be obtained using macromolecular polymers incorporating many streptavidin and peroxidase molecules.

Binding reagents

ANTIBODIES are used in the vast majority of assays as they can provide the levels of SPECIFICITY and SENSITIVITY required. Binding proteins and RECEPTORS are used on occasions when suitable ANTIBODIES are not available [1]. ANTIBODIES are either monoclonal or polyclonal. Polyclonal ANTIBODIES are produced entirely in animals, particularly rabbits. However, an animal's IMMUNE SYSTEM generally produces a rather HETEROGENEOUS mixture of ANTIBODIES. For this reason and for continuity of supply, MONOCLONAL ANTIBODIES are often favoured. Monoclonal production is initiated in mice, but when an ANTIBODY response is observed, their spleens are removed and the suspended spleen cells fused with a myeloma cell line. The fused cell hybridomas are grown in culture; if any culture is positive, it is plated out so that each well contains a single cell. This produces cells that are derived from a single progenitor and gives rise to a single species of ANTIBODY.

Polyclonal ANTIBODIES tend to be of high avidity and can be very specific. However, their exact composition will vary from bleed to bleed, even in the same animal. For this reason it is difficult for commercial kit manufacturers to ensure complete product homogeneity over the lifetime of a commercial

immunoassay. Polyclonal ANTIBODIES tend to be used in competitive assays, which require high-AFFINITY ANTIBODIES and do not consume a lot of ANTIBODY. MONOCLONAL ANTIBODIES tend to be of rather lower AFFINITY but provide a more HOMOGENEOUS reagent. They also have a more closely defined SPECIFICITY. These ANTIBODIES tend to be used in immunometric assays, often in combination with a polyclonal. Finally it should be admitted that ANTIBODY production is more of an art than a science.

Standards

Each time the concentration of an ANALYTE is determined in a sample, it is necessary to prepare a standard curve containing known concentrations of the ANALYTE. The standard is the most important component of an immunoassay. Any error in the standard will produce an error in the estimated ANALYTE concentration. The standard should resemble the ANALYTE as closely as possible. This may seem a rather obvious statement to make, but is often difficult to achieve in practice. For instance, RECOMBINANT proteins are often used as standards. Do they have the same conformation and degree of glycosylation as the native molecule? Standards are preferably calibrated against some type of agreed international standard. Commercial companies also have strict internal quality control criteria to ensure that their kit standards do not fall outside tight performance specifications.

Buffers

A multitude of buffers have been employed in immunoassays, although most often phosphate or Tris buffers at near to physiological pH are used. The buffers usually contain a protein additive to reduce non-specific binding to tube or microtitre plate walls. In addition buffers may contain a bacteriostat to prevent bacterial contamination. One difficult problem often encountered in setting up an immunoassay is related to the different composition of the sample and the standard. This can cause problems during assay validation. Buffers often contain additives such as animal proteins in an attempt to minimise such matrix effects.

Separation systems

Many techniques have been employed to separate ANTIBODY-bound ANALYTE from free ANALYTE. Activated charcoal is often used with tritium tracers. The charcoal selectively adsorbs the free TRACER but is unable to bind the ANTIBODY-bound fraction. The charcoal-bound fraction is separated from the free fraction by centrifugation. Precipitation procedures are popular with ^{125}I tracers. These methods often employ a second ANTIBODY specific for the first to form an immune complex, which can again be separated from the unbound TRACER by centrifugation.

These precipitation techniques have been largely superseded by solid-phase techniques, where either the primary or secondary ANTIBODY is bound to a solid phase. Coated particles are widely used with ^{125}I tracers. Separation of the bound and free fractions can either be achieved by centrifugation or preferably by magnetisation if a magnetisable component is incorporated into the particle. Coated-well techniques have become increasingly popular as the trend away from radioactivity has gained momentum in the immunoassay field. Microtitre plates provide a very convenient format for performing enzyme immunoassays. The ANTIBODY is adsorbed to the walls of the plastic wells and separation of the bound from the free fraction is very readily achieved by washing the plates. This is much more convenient than centrifugation methods.

Data presentation and curve plotting

Many approaches have been used for data plotting and standard curve fitting. One approach is to calculate the binding of TRACER in the standard tubes as a percentage of the binding in the absence of standard. This is then plotted against the log of the standard concentration. A log plot spreads out the data points and makes manual calculation of sample concentrations easier (Fig. 3). A number of alternative curve plotting methods are illustrated in

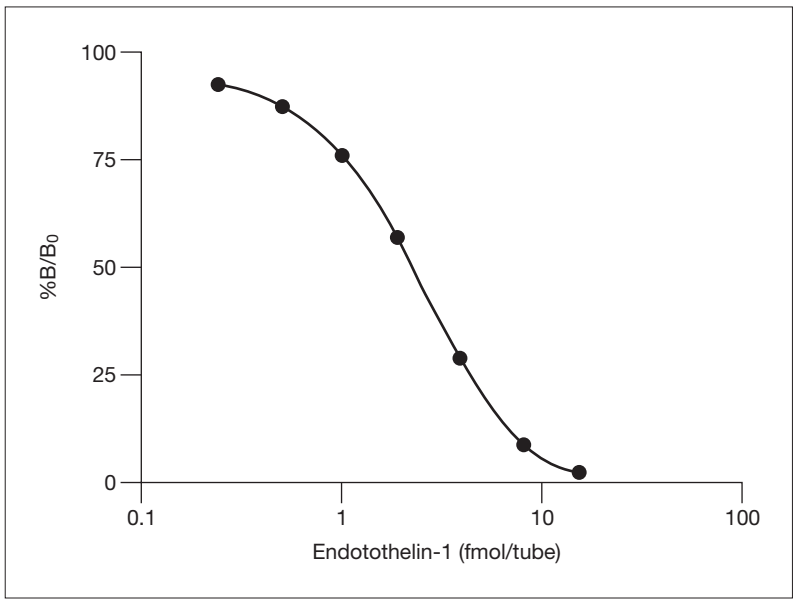

FIGURE 3. A TYPICAL ENDOTHELIN-1 RADIOIMMUNOASSAY STANDARD CURVE

Figures 5–7. The choice of curve fit software can generate a lot of discussion. Whatever method is chosen, it is important to plot out the data and demonstrate that the curve does actually fit the data points.

Selected immunoassays

Endothelin-1 radioimmunoassay (RIA)

This assay has been selected as an example of a competitive assay using an ^{125}I TRACER and magnetic separation. ENDOTHELIN is a potent vasoconstrictor produced by vascular endothelial cells. It produces a strong and sustained vasoconstriction in most arteries and veins of many mammalian species.

Assay protocol

The assay is performed in polypropylene tubes. Standard (100 µL) or sample (100 µL) and antiserum (100 µL) are added and the tubes incubated at 2–8°C for 4 hours. TRACER (100 µL ^{125}I-labelled ENDOTHELIN-1) is then added and the tubes left overnight at 2–8°C. Amerlex-M (250 µL of magnetisable solid particles) is added and left at room temperature for 10 minutes. The ANTIBODY-bound fraction is separated by placing the tubes on a magnetic rack for 15 minutes and then pouring off the supernatant, which contains the free phase. The tubes are then counted for 1 minute in a gamma scintillation counter. In these assays, samples and standards are usually assayed in duplicate.

Cyclic AMP scintillation proximity assay

CYCLIC AMP is a member of a biologically important class of molecules termed "second messengers". This is a term for molecules, which are able to transmit intracellularly the biological effects of compounds not able to enter the TARGET cell themselves. The cAMP assay is an example of a HOMOGENEOUS competitive immunoassay, i.e. an assay in which the bound TRACER does not need to be physically separated from the free fraction. This greatly simplifies the assay and makes it more amenable to automation. It is based on the principle that relatively weak beta emitters such as tritium and the ^{125}I Auger electron need to be close to scintillant molecules to produce light; otherwise the energy is dissipated and lost to the solvent. This concept has been used to develop

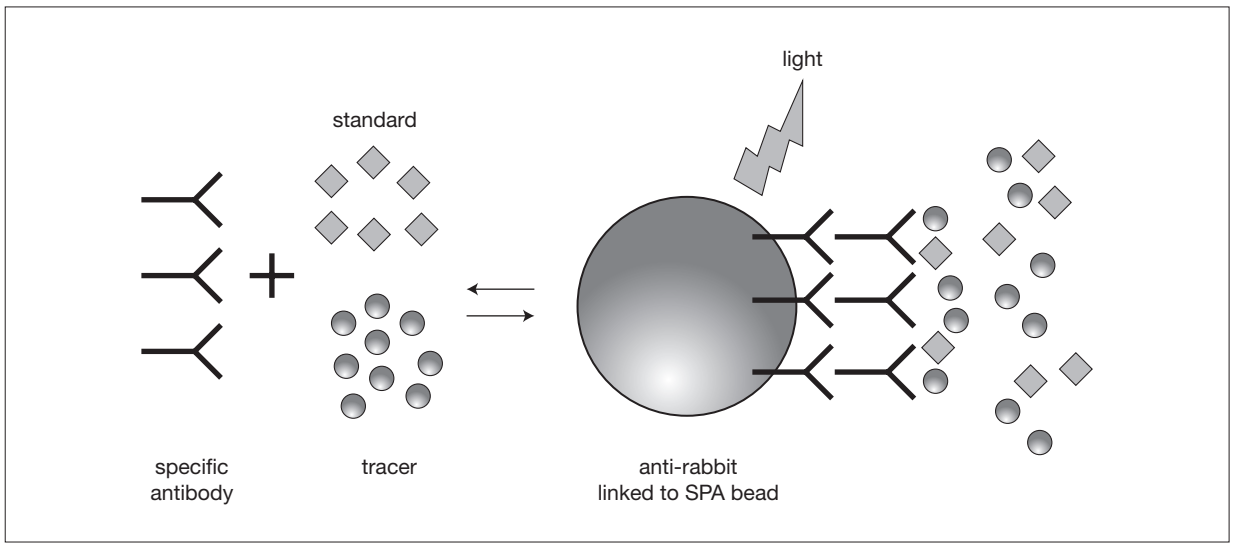

Figure 4. The principle of a SPA immunoassay

HOMOGENEOUS RIAs by coupling second ANTIBODIES onto fluomicrospheres containing scintillant. When a second ANTIBODY-coupled fluomicrosphere is added to an RIA tube, any radiolabelled LIGAND that is bound to the primary LIGAND-specific ANTIBODY will be immobilized on the fluomicrosphere. This will bring into close proximity the radiolabel and the scintillant, activating the scintillant to produce light. Any unbound radioligand remains too distant to activate the scintillant (Fig. 4).The signal is measured in a liquid scintillation counter and is inversely proportional to the concentration of LIGAND in the sample or standard [6].

Assay protocol

Typically, cAMP assays are carried out according to the method of Horton and Baxendale [6].Briefly,50 µL standard or sample followed by 50 µL ^{125}I-labelled cAMP, 50 µL antiserum and 50 µL scintillant beads are pipetted into each assay tube and incubated at room temperature overnight. The amount of TRACER bound to the beads is determined by counting for 2 minutes in a beta scintillation counter. A typical cAMP SPA plot is shown in Figure 5. The data are represented as a log/linear plot.

Leukotriene $C_4/D_4/E_4$ enzyme immunoassay system

The peptido-LEUKOTRIENES comprise the slow-reacting substances of ANAPHYLAXIS.They are potent mediators of bronchoconstriction, vascular and non-vascular smooth muscle contraction, increase vascular permeability and epithelial mucous secretion. They are widely considered to be important mediators in ASTHMA and antagonists to these compounds are being developed as possible anti-ASTHMA drugs. This assay has been selected as an example of a competitive immunoassay using an enzyme LABEL.

Assay protocol

The assay is performed in a 96-well ANTIBODY-coated microtitre plate. Standard or sample (50 µL) is pipetted into each well and incubated at 4–10°C for 2 hours. LEUKOTRIENE C_4-horseradish peroxidase conjugate (50 µL) is then added and incubated for a further 2 hours at the same temperature. All wells are washed thoroughly and 150 µL SUBSTRATE solution added to each. The plate is incubated at room temperature with shaking for 30 minutes and the reaction is then terminated with 100 µL 1 M sulphuric

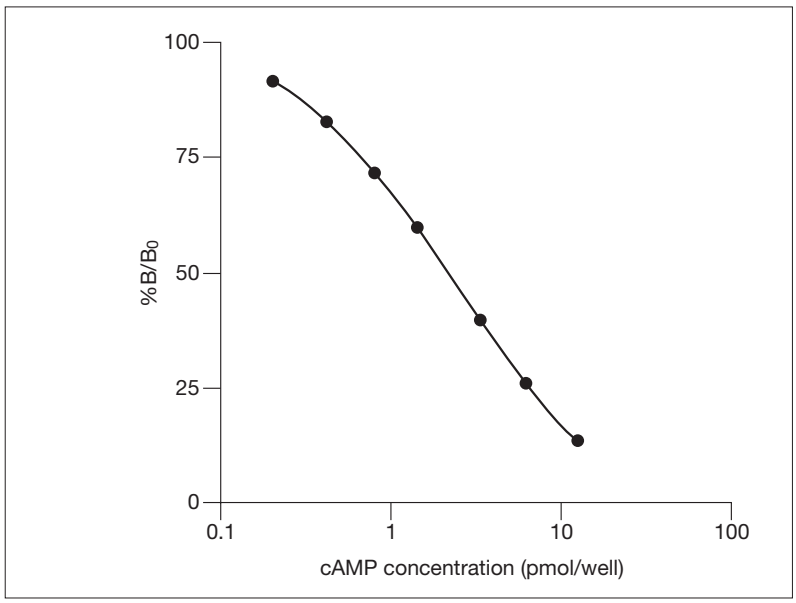

Figure 5. A typical cAMP SPA standard curve

acid. The optical density of each well is determined in an automatic plate reader at 450 nm. A typical LEUKOTRIENE C_4 plot of optical density against the log concentration is shown in Figure 6.

Interleukin-10 ELISA system

INTERLEUKIN (IL)-10 is a glycoprotein that inhibits cytokine synthesis by the Th1 sub-population of T cells. The Th1 CYTOKINES are responsible for many aspects of CELL-MEDIATED IMMUNITY, so IL-10 has immunosuppressive activity. There is considerable interest in investigating the use of IL-10 in transplantation, RHEUMATOID ARTHRITIS and septic SHOCK. This assay has been chosen as an example of an immunometric assay using a biotin-labelled ANTIBODY in combination with a streptavidin/horseradish peroxidase TRACER.

Assay protocol

The assay is performed in a 96-well ANTIBODY-coated microtitre plate. Assay buffer (50 µL) and either standard or sample (50 µL) are added to each well. The plate is incubated at room temperature for 3 hours and then washed. Biotinylated detection ANTIBODY (50 µL) is added to all wells, incubated at room temperature for 1 hour and the plate is washed. Streptavidin/horseradish peroxidase (100 µL) is then added, incubated for 30 minutes and the plate is washed again. SUBSTRATE solution (100 µL) is then added and incubated for a further 30 minutes. The reaction is then terminated with 100 µL dilute sulphuric acid and the optical density measured at 450 nm. The data are represented as a log/log plot. A typical IL-10 log/log plot analysed by linear regression is shown in Figure 7.

Assay performance and validation

When either developing or evaluating an immunoassay, a number of questions relating to the performance of the assay need to be considered. These include the likely cost of the assay, how easy is it to perform, the equipment required to carry out the assay, what ANALYTE concentration can be measured, how reproducible the assay is and whether or not the assay measures the true ANALYTE concentration. Some of these questions have already been covered. The remainder is discussed in the following sections.

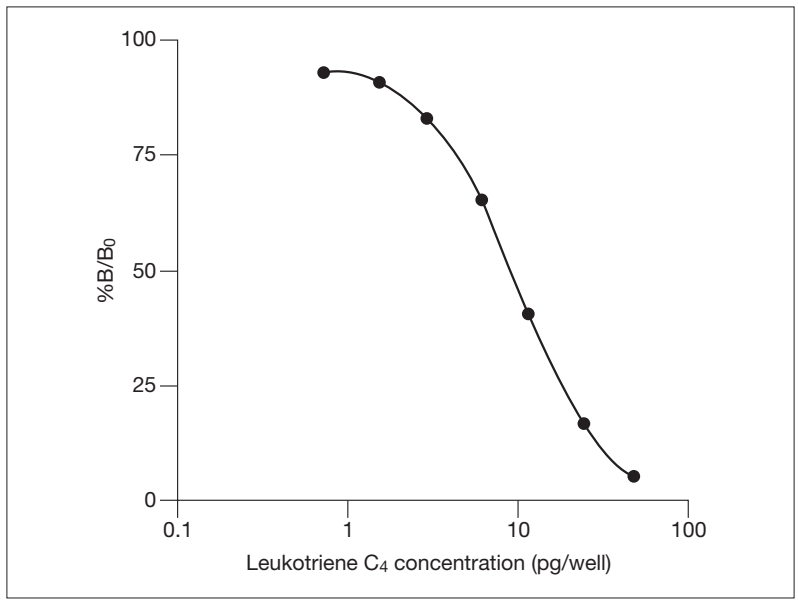

FIGURE 6. A TYPICAL LEUKOTRIENE C_4 ENZYME IMMUNOASSAY STANDARD CURVE

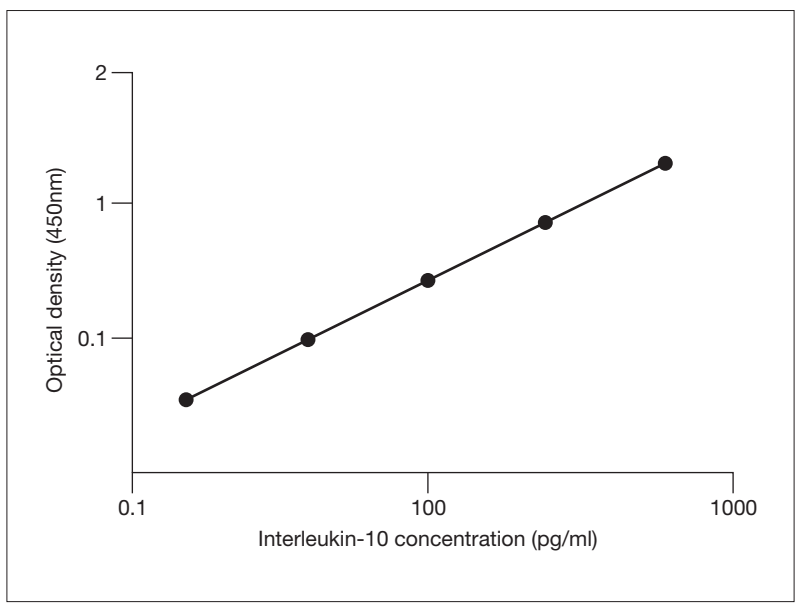

FIGURE 7. A TYPICAL INTERLEUKIN-10 ELISA STANDARD CURVE

Assay precision

Precision is an index of assay reproducibility and is a guide to how much the determined ANALYTE concentration is likely to vary from measurement to measurement. Within-assay and between-assay precision refers to the reproducibility of measurement in single and multiple assays, respectively. Precision is likely to vary throughout the standard curve range. A precision profile of the assay can be constructed by

performing multiple measurements at each standard concentration. It is difficult to state what is acceptable with regard to assay precision, as this will vary depending upon the intended application of the assay. A reasonably well-designed assay will have a within-assay precision of <10% at the extremities of the standard curve and <5% over most of the assay. The between-assay precision is usually a few percent higher than the within-assay precision. In a well-designed assay, the sample concentrations will normally fall within the part of the standard curve having the highest precision.

Assay sensitivity

The SENSITIVITY of an assay is the lowest level of ANALYTE that can be detected. Various ways of calculating assay SENSITIVITY have been used. One common method is to calculate the standard deviation of the zero standard and express the SENSITIVITY as that value corresponding to two standard deviations from the zero standard. However, it is important to be aware that samples cannot be assigned a PRECISE value near to the SENSITIVITY limit of an assay, as the precision at these concentrations will be extremely poor. Samples should only be given values when they fall within the range of the standard curve data points. The range of the data points should be set from the precision profile. Any sample values outside the standard curve range should really be given the value of less than or greater than the lowest or highest standard, respectively.

Assay validation

Before using an immunoassay it is important to validate the assay. An assay should be validated for each SAMPLE MATRIX such as plasma, serum or cell culture supernatant that will be used in the assay. The SPECIFICITY of an assay is confirmed by testing against related substances. The analytical recovery is assessed by adding known amounts of the ANALYTE to the SAMPLE MATRIX under evaluation and measuring the percentage recovery of the ANALYTE. Assay linearity is determined by diluting samples and determining whether or not the measured values are in agreement with the non-diluted sample when the dilution factor is taken into account.

In some situations it can prove difficult to develop a valid assay without some kind of sample purification step. This is exemplified when preparing cellular extracts for cAMP measurement. Traditionally these involve tedious processes, such as acid or solvent extraction, to obtain samples in a suitable form for subsequent assay. A direct assay method has been more recently developed eliminating more difficult sample extraction methods for cAMP. This latter method enables the culture of cells followed by a simple, direct extraction and assay method for cAMP to be achieved with little technical intervention [1].

Summary

Immunoassays have remained a core technique in the biological sciences for the last 40 years. It is interesting to speculate on future developments in the field. It is easy to predict that the range of ANALYTES measured by immunoassays will continue to grow. For example, immunoassays for CYTOKINES and METALLOPROTEINASES will continue to be developed as new molecular species, or biomarkers, are identified. The way in which the technology will develop is more difficult to foresee. One can predict that there will be three trends, a move towards high-throughput assays, the MULTIPLEXING of assays, and to the widespread use of highly simplified, home or point-of-care tests.

Automation will become even more widespread in the clinical field and high-throughput pharmacological screening in the pharmaceutical industry. There is likely to be an increased reliance on fluorescent and luminescent detection systems [7] due to the SENSITIVITY and ease of use of such labels. The format of the assays will move from a 96- to a 384- and possibly 1536-well plate design. These plate designs will increase assay throughput and decrease reagent costs. The drive towards assay miniaturization, high assay throughput and the use of either fluorescence or chemiluminescence detection systems is changing the way researchers are detecting assays. Charged-coupled devices (CCD) such as

LEADseeker™ Multimodality Imaging System are capable of imaging any microtitre plate footprint in seconds [8]. The same requirements for high-throughput assays are motivating a trend towards the development of HOMOGENEOUS assays. The lack of a separation step means that these systems are much more amenable to automation [9].

A second broad emerging trend is the development of multiplexed assays. In this approach, multiple ANALYTES are measured in one assay [10]. Commercial kits such as the SearchLight™ system are available. This technology resembles a traditional "sandwich ELISA" except that a grid of between 9 and 25 capture ANTIBODIES is arrayed on the base of each well of a 96-well microtitre plate. The assay is performed in a similar fashion to a conventional ELISA except that all the ANALYTES can be simultaneously quantified in each sample. ANALYTE binding is detected using an enzyme LABEL and a chemiluminescent SUBSTRATE. The signal from each spot within the well is quantified using a CCD instrument. The SearchLight procedure in combination with the LEADseeker imager has been used to quantify nine CYTOKINES simultaneously in each sample. The SENSITIVITY and standard curve range are superior to conventional cytokine ELISAs.

An alternative approach to MULTIPLEXING has been developed by the Luminex Corporation. Luminex internally dye polystyrene microspheres with two spectrally distinct fluorochromes. Using varying ratios of these two fluorochromes, 100 spectrally distinct microspheres sets have been developed. These microspheres allow the MULTIPLEXING of up to 100 different ANALYTES. A third FLUOROCHROME coupled to a reporter molecule (an adaptation of the assay principle outlined in Fig. 2) quantifies the extent of the reactions taking place at the surface of these microspheres. The assay is quantified in a specially designed FLOW CYTOMETRY-like instrument equipped with two lasers. One laser identifies the microspheres (so identifying the ANALYTE); the second quantifies the amount of the fluorochromes bound to each microsphere (so quantifying the ANALYTE). This system has been used to multiplex a variety of ANALYTES [11]. This method has been used to set up multiplexed assays of four CYTOKINES in both HOMOGENEOUS and HETEROGENEOUS format using a fluorescent phycoerythrin-based detection reagent. The HETEROGENEOUS assays have a similar SENSITIVITY and a wider standard curve range compared to conventional cytokine ELISAs.

Immunoassays have been a core analytical technique in the biological sciences for more than 40 years. The continual improvement in the methodology and the development of novel approaches, particularly with point-of-care and self-test methods, will ensure that the technology will continue to play a central analytical role for the foreseeable future.

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Flow cytometry

John F. Dunne and Holden T. Maecker

Introduction

Various technologies exist for analyzing cells in suspension using optical or electrical interrogation techniques. This chapter focuses on flow cytometers, tools that have optimized fluidics, electronics and optics to generate extraordinary measurement precision and high dimensionality on samples of cells moving through an image plane. Other devices, including scanning cytometers, optical microscopes, and counting devices based on electrical impedance measurements, are outside the scope of this discussion.

While the fundamental fluidics constraints of flow cytometers have not changed, the equipment available to analyze cells as they pass through a flow cell has been enhanced dramatically with developments in lasers, digital electronics, fluorescence chemistries and computer power. Analysis rates have migrated from hundreds to tens of thousands of cells per second, but more importantly, the number of colors that can be simultaneously measured has gone from 1 or 2 in the 1970s, to 12 or more in recent years [1]. These polychromatic experiments create a new level of information about blood cells, as well as a new set of technical challenges. The optimization of instrument and experimental protocols and the efficient analysis of this kind of data become critical to successful implementation of this technology.

Mechanistic principles

Fundamentally, flow cytometers measure fluorescent or scattered light emitted by a cell during its illumination in bright light, typically a highly focused laser beam [2]. In most cases, the cell stream is positioned using hydrodynamic focusing, in which a thin core of cell suspension is limited to the center of a larger flowing sheath fluid. The cells thus arrive sequentially into the laser beam at thousands of cells per second.

Light scatter measurements

Light is scattered by the cells intersecting the laser, and this scattered light is usually measured at narrow angles just above and below the laser beam (commonly called "FORWARD SCATTER"), and by a separate detector at wider angles orthogonal to the beam (commonly called "SIDE SCATTER"). FORWARD SCATTER is descriptive of the size of the cell, while SIDE SCATTER is proportional to size and granularity. Thus, in commonly analyzed populations of blood cells, platelets, LYMPHOCYTES, MONOCYTES, and GRANULOCYTES can be distinguished reasonably well simply on the basis of their intrinsic light scattering properties (Fig. 1).

Fluorescence measurements

More valuably, cells can be stained with fluorescent reporter molecules, and the binding of these reporters can describe extremely subtle phenotypes. The most common class of such reporters comprises fluorescently conjugated MONOCLONAL ANTIBODIES. Thousands of these products are commercially available. The ANTIBODY binds very specifically to particular EPITOPES in proteins present on the cell surface, or if the cell is permeabilized the ANTIBODY can enter the cell and bind to EPITOPES within. Since the ANTIBODY is covalently linked to a fluorescent molecule, the ANTIBODY binding is directly correlated to the fluorescent

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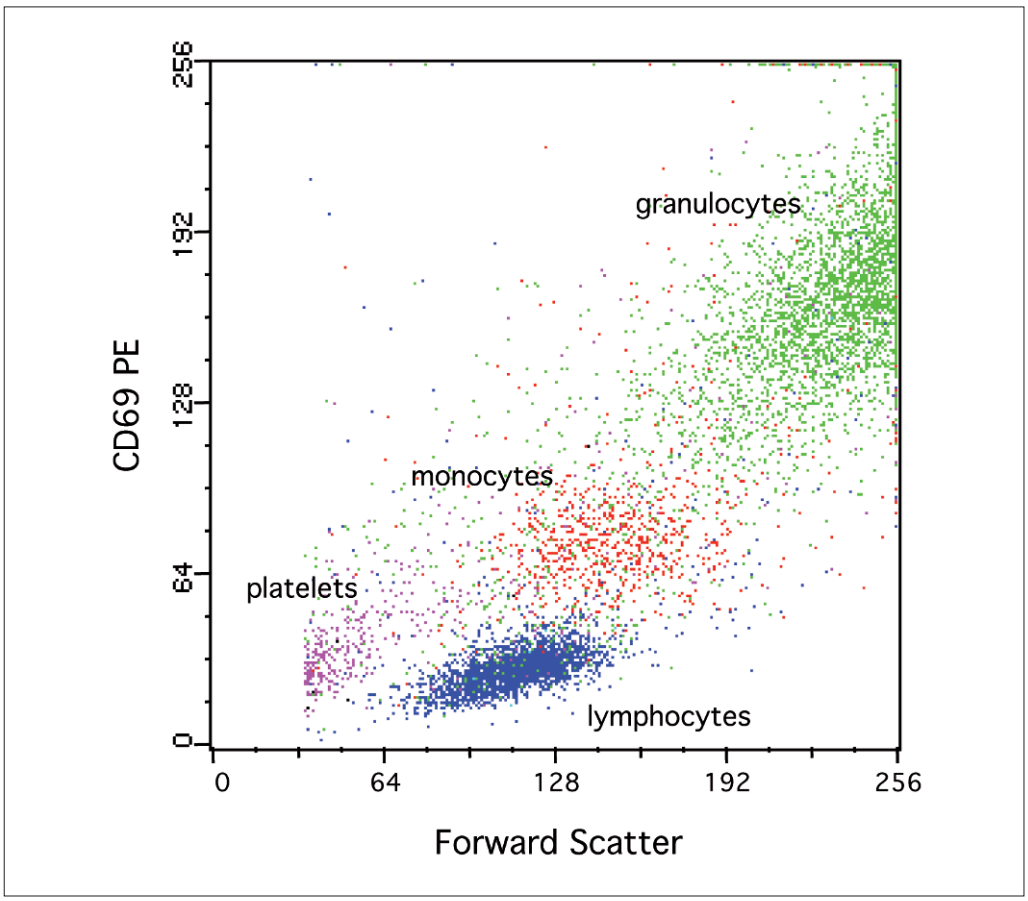
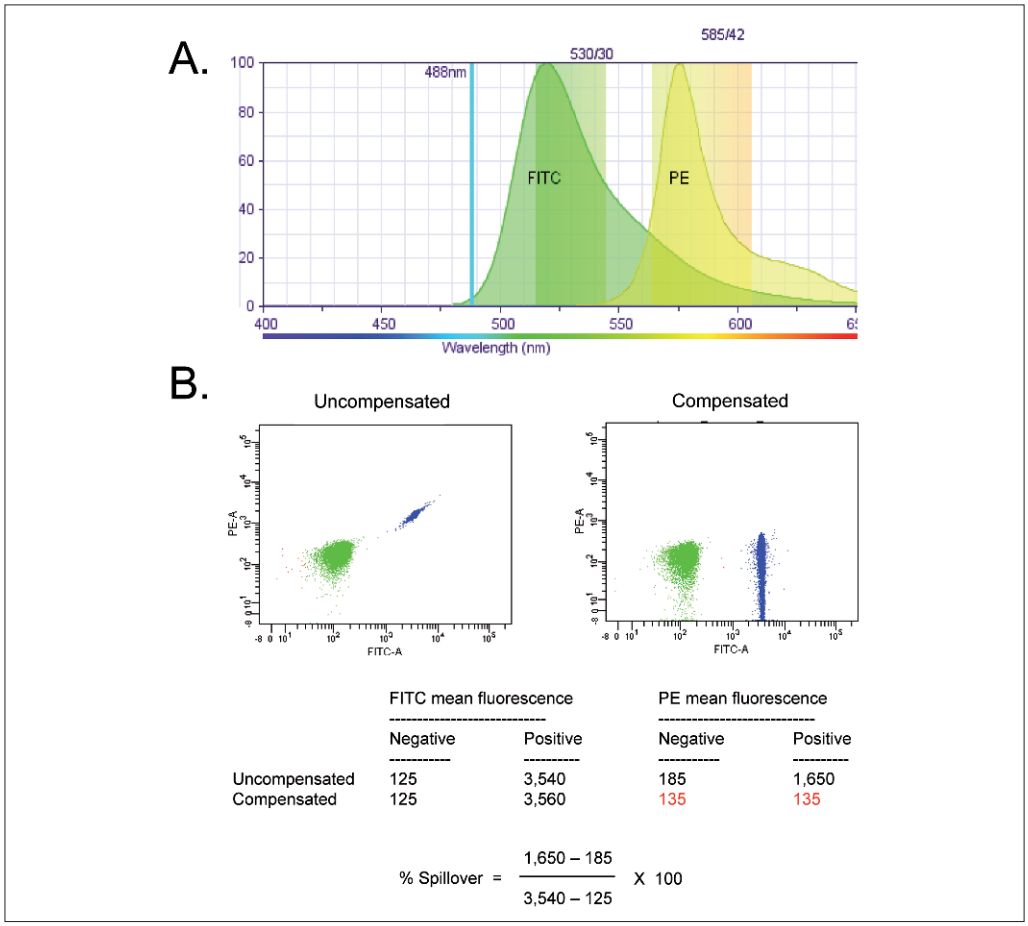


Figure 1. Discrimination of leukocyte subsets by light scatter
The labeled subsets of leukocytes were fluorescently stained with specific monoclonal antibodies (not shown), and the specific antibody-stained populations were colored as indicated. These populations were then displayed in a dot plot of forward versus *side scatter. A threshold was set on forward scatter, excluding much of the platelet population to prevent the collection of small debris that would otherwise interfere with the analysis. Note that the colored populations are reasonably well resolved from each other. However, the relative positions of these populations will depend upon the treatment of the sample (in this example, formaldehyde-fixed and detergent-permeabilized cells were used).*

signal emanating from the cell during laser excitation. In a well-developed assay, the brightness associated with ANTIBODY binding can be a direct measure of the abundance of the relevant antigenic protein (see Box 1). By using several MONOCLONAL ANTIBODIES, each conjugated to fluorescent dyes with characteristic colors, multiple populations of cells can be identified in a single sample, using optical detectors that filter light to detect only specific wavelengths.

Fluorescence spillover and compensation

Fluorescent reporter molecules, whether small organic dyes or larger protein molecules, have characteristic excitation and emission curves within the optical spectrum. Because these curves are relatively broad, overlap is inevitable, and leads to detection of more than one FLUOROCHROME per optical detector. This is referred to as "spillover" (Fig. 2). To properly decon-



	FITC mean fluorescence		PE mean fluorescence	
	Negative	Positive	Negative	Positive
Uncompensated	125	3,540	185	1,650
Compensated	125	3,560	135	135

$$\% \text{ Spillover} = \frac{1{,}650 - 185}{3{,}540 - 125} \times 100$$

Figure 2. Spectral overlap and optical spillover

(A) Detection of FITC in the PE detector is a consequence of the overlapping emission spectra of FITC and PE. (B) By calculating the signal in both the FITC and PE detectors from a sample stained only with FITC, the relative spillover can be calculated, and a compensation factor applied, so that singly stained populations in fact appear so in the data display.

Box 1. Why use flow cytometry as an analytic method?

Many different methods exist for quantifying the proteins expressed by a population of cells. In addition to flow cytometry, Western blotting, immunoprecipitation, ELISA, RIA, enzyme-linked immunospot (ELISPOT), fluorescence microscopy, and immunohistochemical staining are all methods used to quantify cellular proteins. Of these, only flow cytometry, ELISPOT, fluorescence microscopy, and immunohistochemical staining provide information on a single-cell basis. The others are bulk assays that do not quantify the number or phenotype of cells that are expressing the protein of interest (though they may provide other information, like the size of the targeted protein). Of the single-cell assays, flow cytometry is unique in the number of fluorescence parameters that have been combined in a single assay. Thus, it provides the richest information, on a per-cell basis, of the current generation of assays for cellular protein analysis.

volute the spillover, fluorescence "COMPENSATION" is applied to the signals in multicolor experiments. Most commonly, a spillover matrix is calculated based on the observed fluorescence in all detectors of samples each stained with a single FLUOROCHROME. Such samples can be single-stained cells, ANTIBODY capture beads, or dye-embedded beads. The inverse of the spillover matrix thus calculated becomes the COMPENSATION matrix, which is applied to all experimental sample measurements. In newer digital flow cytometers, these calculations are automated and are done using a software algorithm (referred to as "software COMPENSATION"). This has the advantage that one can view and change the applied COMPENSATION at any later time during the analysis of the data. Earlier analog systems calculated COMPENSATION during the actual fluorescence measurement, and stored only the compensated result (referred to as "hardware COMPENSATION"). Besides inaccuracies in the way these systems calculated COMPENSATION, the limitations of hardware COMPENSATION in terms of checking and correcting potential COMPENSATION errors are significant.

Classification/types of assay

Immunophenotyping

In the 1980s, with the early propagation of FLOW CYTOMETRY and MONOCLONAL ANTIBODIES, there was some expectation that the protein surfaces of hematopoietic cells could be mapped with ever-increasing complexity to reveal changes in the types of cells present, or their relative abundance, that might correlate with clinical disease onset or progression. In the intervening 30 years, with thousands of fluorescent ANTIBODIES commercially available, the management of only two patient classes is substantially driven by these kinds of flow assays: HIV disease and leukemia/lymphoma.

Flow immunophenotyping in HIV disease

One of the most proximal and dramatic effects of HIV INFECTION is an eventually lethal loss of $CD4^+$ T cells [3]. This was one of the first easily recognized manifestations of infection, indeed the first clinical test that helped identify AIDS patients [4], and has been one of the most useful analytical tools available to characterize mechanisms of HIV pathology as well as therapeutic benefit of anti-HIV treatment. Often augmented by assays that quantitate viral load, CD4 counting remains the standard method of monitoring disease progression [4].

Among the FLOW CYTOMETRY assays, the CD4 counting assay is relatively simple. Usually, fluorescently conjugated CD4 MONOCLONAL ANTIBODIES are added to whole blood, generally as part of a cocktail of two to four ANTIBODIES that together uniquely identify those T cells that bear the CD4 antigen. With the addition of a known number of fluorescent beads per volume of blood, the software calculates the ratio of beads to $CD4^+$ T cells. From this, it can report the number of CD4 cells present per microliter of blood. Normal ranges center around 1000 $CD4^+$ cells per microliter. AIDS progression quickly results in levels that are half of that, and profound disease is correlated with CD4 counts at or below 200 $CD4^+$ cells per microliter.

With the increasing availability of antiretroviral therapies in developing countries, the need for low-cost CD4 monitoring that can be robustly carried out in remote settings has become a new challenge. Smaller dedicated flow cytometers with preformatted reagents for CD4 counting are now available, but other technologies are also being tested, including light microscopy and similar techniques requiring minimal technology investment and training.

Flow immunophenotyping in leukemia/lymphoma

The observation that the major immunophenotypes found in peripheral blood are relatively stable may have been disappointing in some regards, but it was helpful in allowing the recognition of unusual phenotypes characteristic of cancer cells. In particular, in the case of transformed cells of hematopoietic lineages, these unusual phenotypes have now been mapped into broadly recognized categories of malignant disease, and flow cytometric assays are thus

useful to help diagnose and monitor leukemias and lymphomas.

Several clinical research consortia have published consensus documents describing the general utility, practical aspects and interpretation of combinations of markers and their distribution on cancer cells [5–7]. However, until now, no commercially available, or otherwise standard, kit has been approved for diagnostic use, and common practice is for clinical centers to use their own discretion to implement and validate such assays. Nevertheless, the standard of care of patients suffering from these malignancies has been improved, sometimes dramatically, based on practitioners' ability to detect and characterize leukemias and lymphomas by FLOW CYTOMETRY.

Immunotoxicology immunophenotyping

While most common disease states do not change the frequency of major blood cell components, the toxicity associated with novel drugs or industrial pollutants can have profound effects. Recent work establishing consensus protocols for the evaluation of immunotoxicity of such chemical entities documents a growing utility for this class of assays [8]. The foci of these assays are typically rodent, dog or non-human primate models, and monoclonal ANTIBODY reagents are less commonly available or less well-characterized. The normal ranges of various lymphoid cells types, other environmental factors that can influence these ranges, and the magnitude of changes in these cell types that should be used as sentinels for concern or prohibit the use of such chemicals in various exposure scenarios, all represent ongoing work.

Functional assays

An important additional class of flow assays consists of those assays that experimentally induce a functional response in cells of interest, then use FLOW CYTOMETRY to measure the response. One of the most common assays of this type is the measurement of cytokine production in LYMPHOCYTES in response to antigenic stimulation. The production of a cytokine can be easily measured using well-established reagents and protocols, and is a hallmark of lymphocyte function. With multicolor FLOW CYTOMETRY, the frequency of responsive cells, the amount and kinetics of cytokine production, and the combination of various CYTOKINES can very richly describe an immunological response qualitatively and quantitatively. These assays are discussed in detail below, and by way of comparison, other functional assays are also overviewed (see Box 2).

Proliferation

One hallmark of lymphocyte function is cellular proliferation. Several flow assays have been established that have been correlated in the literature to traditional [^{3}H]thymidine incorporation associated with DNA synthesis. Perhaps the most directly correlated of these flow assays is the use of bromodeoxyuridine (BrdU), an ortholog of thymidine used by DNA synthase and incorporated in the nuclei of proliferating cells. BrdU is detected by specific FLUOROCHROME-conjugated MONOCLONAL ANTIBODIES after nuclear permeabilization [9].

Another proliferation assay utilizes a cytosolic dye, commonly carboxyfluorescein diacetate succinimidyl ester (CFSE), which is used to LABEL cells prior to *in vitro* stimulation. The dye then becomes diluted with each sequential cell division, resulting in successive generations of cells with half the fluorescence of their parent [10]. By calculating the frequency of cells in each generation, an estimate of the original progenitor frequency can be made. This calculation is flawed by not accounting for cell death. Still, one can differentiate between a large progenitor population that has only divided once or twice, and a small progenitor population that has divided many times. By contrast, this difference would be difficult to see using [^{3}H]thymidine or BrdU incorporation.

One of the earliest flow assays for proliferation, which is still in common use, is the direct measurement of DNA content using a quantitative DNA stain such as propidium iodide or the vital dye Hoescht 33258 [11, 12]. This is especially useful for peripheral blood LYMPHOCYTES, which are natively arrested with a 2c DNA content in the G0 phase of the cell cycle.

Box 2. Different assays for measuring T cell function

In this chapter, measurement of intracellular cytokines by flow cytometry is described in some detail as a method for determining T cell functional responses after short-term stimulation. However, alternative methods are also in common use. Some are flow cytometry assays, such as MHC-multimer staining [41], or cytokine capture assays [42]; others use different analytical platforms, such as ELISPOT [43]. The main advantage of flow cytometry as an analytical platform is its multiparameter capability (see Box 1). Of the flow cytometry methods available for measuring T cells in a short-term assay, MHC-multimer staining is unique in that it measures T cell specificity rather than function, and thus requires no activation at all. Multimeric forms of MHC molecules with bound antigenic peptides are produced and labeled with a fluorochrome such as PE. These can then be used to stain T cells (via their T cell receptor) in much the same way as a fluorochrome-conjugated antibody. However, their use requires a knowledge of the particular peptide and MHC restriction pattern of a T cell immune response; as such, they are not useful for quantitating the overall T cell response to a pathogen, especially in an MHC-diverse population. Cytokine capture assays, like intracellular cytokine staining, measure responses without regard to MHC restriction. The cytokine capture assay is especially useful if one wishes to maintain the cells in a viable state for sorting and further analysis (since it does not require fixation and permeabilization). Still newer assays, such as measures of granule exocytosis [44], can also be used on viable cells, and add to the armamentarium of tools available for dissecting T cell responses.

Upon stimulation, they enter S phase, and progress to the 4c DNA content typical of G2/M. This doubling of DNA is easily recognized flow cytometrically, and the percentage of cells with more than 2c DNA can be roughly related to traditional metrics like mitotic index.

Another nucleic acid stain, acridine orange, has the unusual property of shifting its emission spectrum depending on whether it is bound to single- or double-stranded nucleic acid (roughly speaking, RNA or DNA) and has been used to simultaneously report DNA doubling and the increase in mRNA characteristic of lymphoid stimulation [13].

Phosphoepitope flow cytometry

One intriguing class of functional assays is the quantitation by FLOW CYTOMETRY of signaling molecule phosphorylation states. This has been made possible by the development of MONOCLONAL ANTIBODIES specific for particular phosphoepitopes of common signaling intermediates. Using optimal permeabilization strategies, these phosphoepitopes can be exposed for intracellular staining by the relevant ANTIBODIES, in combination with staining for phenotypic markers or cell subsets [14]. While not yet clinically approved, such assays have been shown to be useful in predicting disease outcome for certain types of cancers [15]. They can also be used to test the potency of kinase inhibitors *in vitro* [16] or in clinical trial monitoring.

Bead matrix immunoassays

Recently bead-based immunoassays have been developed for flow cytometric assessment of various soluble ANALYTES. Commonly, each member of a set of beads is identified by specific fluorescence levels and/or size, and acts as an ANALYTE capture platform using specific covalently bound MONOCLONAL ANTIBODIES. The amount of captured ANALYTE is measured using a second ANTIBODY specific for an alternate site of the same ANALYTE, this second ANTIBODY being conjugated to a fluorescent reporter. Since the various beads can be recognized independently, and since each captured ANALYTE can be quantitated independently, this assay format is well suited to multiplexed analysis. Thus, small volumes of biological fluids (e.g., as little as 10 μl of tears [17]) can be inspected for quantitation of soluble proteins or other ligands

for which specific ANTIBODY pairs are available. Common implementations of this strategy include the simultaneous quantitation of 5–50 different immunomodulatory proteins including CYTOKINES, CHEMOKINES, GROWTH FACTORS and hormones. Commercially available kits range from completely integrated systems to basic platforms that can be developed for custom ANALYTE sets, and are used broadly in basic research and drug discovery PROTEOMICS projects. However, as yet, no patient management tools in this class have yet been approved for *in vitro* diagnostic use.

Components/construction of assays

Traditionally, FLOW CYTOMETRY sample preparation and processing has been constrained by the fact that flow cytometers were designed to accept tubes, and those tubes were loaded onto the instrument manually, one at a time. A second constraint came from instrument set-up and data analysis, which were typically time consuming and required a certain knowledge base. Both of these constraints are now changing with new instrumentation and software. Current cytometers can often handle racks of tubes that are automatically run in a walk-away mode. Plate loaders are also available that can feed samples from 96-well plates directly to the cytometer, again in a walk-away mode. These developments have been complemented by software that can either: (1) perform data analysis simultaneously with acquisition; or (2) perform a batch analysis routine that analyzes all of the data from an experiment at once. The usefulness of such analysis routines is further augmented by flexible analysis templates that can accommodate changing data without repetitive adjustment by the operator [18].

The impact of these changes in FLOW CYTOMETRY hardware and software have opened up the use of FLOW CYTOMETRY to larger pre-clinical and clinical studies that might involve hundreds of specimens. However, the steps involved in sample preparation can still be complex, as detailed below. Automation of these steps will further allow the use of FLOW CYTOMETRY in high-volume settings where it was previously considered too cumbersome.

Antibodies for cell staining

Since the late 1970s, polyclonal antisera have been increasingly replaced by MONOCLONAL ANTIBODIES for most immunological applications, including FLOW CYTOMETRY. MONOCLONAL ANTIBODIES can be produced in unlimited quantities, have better lot-to-lot reproducibility, and tend to have lower backgrounds than polyclonal antisera [19].

Another trend in FLOW CYTOMETRY has been the increased use of direct FLUOROCHROME conjugates, rather than indirect fluorescence analysis using second-step ANTIBODIES or other reagents that carry the FLUOROCHROME tag. While indirect staining can sometimes amplify the fluorescence signal compared to direct staining, it may be at the expense of increasing background fluorescence [20]. Also, multiparameter FLOW CYTOMETRY is much more difficult using indirect staining methods, due to the potential for interaction between multiple second-step conjugates and the primary ANTIBODIES.

Some of the more common fluorochromes used in FLOW CYTOMETRY today are listed in Table 1. They consist of small organic dyes, larger proteins with fluorescent moieties, and nanocrystals referred to as quantum dots (Qdots) [21]. Qdots are rather unique in their relatively sharp emission peaks, and are excited by a range of wavelengths, most efficiently in the violet region of the spectrum. Not all fluorochromes are compatible with all cytometers, or even with all other fluorochromes, as can be predicted from their excitation and emission spectra (see for example www.bdbiosciences.com/spectra). Minimizing spillover between detectors is a major consideration when choosing fluorochromes for multicolor experiments. Relative brightness and compatibility with certain experimental parameters (e.g., permeabilization protocols) will also steer the choice of fluorochromes. For a more complete discussion of the issues of designing multiparameter experiments, see [22–24].

Cell types

FLOW CYTOMETRY can be performed on any cell type that can be rendered into a single-cell suspension.

Table 1. Commonly used fluorochromes for antibody-coupled flow cytometry

Fluorochrome	Type of molecule	Typical excitation laser (nm)	Approximate emission peak
Fluorescein isotyocyanate (FITC)	Small organic	488	518
AlexaFluor 488	Small organic	488	518
Phycoerythrin (PE)	Protein	488 or 532	574
PE-Texas Red	Protein tandem	488 or 532	615
PE-Cy5	Protein tandem	488 or 532	665
Peridinin chlorophyll protein (PerCP)	Protein	488 or 532	676
PerCP-Cy5.5	Protein tandem	488 or 532	695
PE-Cy7	Protein tandem	488 or 532	776
Allophycocyanin (APC)	Protein	633	659
AlexaFluor 647	Small organic	633	667
AlexaFluor 700	Small organic	633	718
APC-Cy7	Protein tandem	633	784
Pacific Blue	Small organic	405	454
AmCyan	Protein	405	487
Pacific Orange	Small organic	405	
Quantum dot (Qdot) 605	Molecular nanocrystal	405	605
Qdot 655	Molecular nanocrystal	405	655
Qdot 705	Molecular nanocrystal	405	705

Since blood cells already exist in this state, they have been most widely studied by the technique. However, adherent cells or tissues can also be analyzed if they are dissociated from each other and/or their SUBSTRATE using either enzymatic (e.g., trypsin, accutase) or non-enzymatic (e.g., EDTA) treatments. Where possible, non-enzymatic dissociation protocols are preferable, because they do not cleave cell-surface proteins that might be targets of the flow cytometric analysis.

Erythrocyte lysis

Whole blood consists of relatively HOMOGENEOUS ERYTHROCYTES, and a much more complex collection of LEUKOCYTES. These can be stained with FLUOROCHROME-conjugated ANTIBODIES in the context of whole blood and resolved based on fluorescence. But analysis of whole blood is difficult because of the light scattering properties of the ERYTHROCYTES, which are so numerous as to obscure the illumination of the LEUKOCYTES. Fortunately, ERYTHROCYTES are differentially sensitive to hypotonic lysis, and can be removed by incubation of blood with ammonium chloride in water. LEUKOCYTES are more resistant to osmotic damage than ERYTHROCYTES, which cannot exclude this salt, and subsequently take up water and are lysed. Typically, whole blood is treated for 10 minutes with a large volume of ammonium chloride solution either before or immediately after staining with FLUOROCHROME-conjugated ANTIBODIES.

An alternative to ammonium chloride lysis involves hypotonic lysis in the presence of a fixative

(e.g., formaldehyde), whereby the ERYTHROCYTES are preferentially lysed while LEUKOCYTES are fixed in a single incubation of 10 minutes or so. Solutions for this procedure are commercially available. After lysis/fixation, the cells may be directly analyzed by FLOW CYTOMETRY ("no-wash" assays), or they may be subjected to washing to remove the residual red cell debris and unbound flurochrome-conjugated ANTIBODIES before analysis ("washed" assays). Washed assays typically allow better signal-to-noise discrimination of fluorescently stained populations, and better visualization of lymphocyte scatter properties. However, no-wash assays have become popular in clinical laboratories because of their simplicity. Cells are detected based on threshold staining for a common leukocyte antigen, such as CD45, and these assays can adequately resolve subpopulations of LYMPHOCYTES.

Density gradient separation

As an alternative to erythrocyte lysis of whole blood, mononuclear LEUKOCYTES (LYMPHOCYTES and MONOCYTES) can be directly isolated from whole blood prior to staining. Solutions of high molecular weight carbohydrates, such as FICOLL, are used for this separation, which is accomplished on the basis of density [25]. By underlaying whole blood (usually diluted 1:1 with buffer or tissue culture media) with a FICOLL solution, a density gradient is formed. This biphasic solution is then subjected to centrifugation (typically at about $400\times g$ for 15–20 minutes). ERYTHROCYTES and GRANULOCYTES, which have the greatest buoyant density, are pelleted to the bottom of the FICOLL layer. LYMPHOCYTES and MONOCYTES, which are less dense, collect at the interface of the plasma and FICOLL, where they are collected by pipetting. Platelets, the least dense LEUKOCYTES, will remain in the plasma layer. Successive centrifugation of the mononuclear cells collected from the interface is then carried out to remove residual FICOLL and further deplete the mononuclear cells of platelets.

Density gradient separation techniques are a standard, albeit time-consuming method for the isolation of mononuclear cells from small to large volumes of blood. However, simpler alternatives are also available that consist of a gel matrix pre-dispensed into a blood collection tube or centrifuge tube. By adding whole blood and centrifuging at a prescribed speed, the mononuclear cell layer can be collected from the top of the gel interface, while ERYTHROCYTES and GRANULOCYTES are forced through the gel plug. Such systems are available from commercial vendors, and provide generally equivalent results with greater convenience than the FICOLL separation technique [26]. Mononuclear cells isolated by either method are often cryopreserved in liquid nitrogen, allowing them to be banked for later studies, including FLOW CYTOMETRY.

Activation

As described briefly above, functional assays are those in which the cell types of interest are stimulated *in vitro* and allowed to manifest some kind of response, the quantity and quality of which is measured in the flow cytometer. Lymphocyte activation is a particularly valuable class of functional assays, and typically requires specific antigen, polyclonal MITOGEN, or a cytokine as a stimulus. Short-term simulation (5–30 minutes) can be optimal for detecting phosphorylation changes in signaling proteins such as Erk, Mek, Akt, the Stat proteins, and a host of other phosphoproteins [14]. Longer stimulation (4–24 hours) can be used to detect cytokine production, such as IL-2, IFN-γ, TNF-α, etc. [27]. Detection of cytokine production is aided by addition of a secretion inhibitor, such as brefeldin A [28] or monensin [29], which allows for intracellular accumulation of the CYTOKINES and thus brighter staining. Finally, stimulation for periods of 2–7 days is typical for detecting cell proliferation, whether using CFSE dye dilution, BrdU incorporation, or other assays.

Fixation/permeabilization

For staining of intracellular EPITOPES, cells are first fixed (usually with formaldehyde) and then permeabilized (usually with detergent, or in some cases methanol). Commercial reagents containing fixatives and detergents are readily available and allow for

reproducible fixation and permeabilization protocols.

Cell staining

For assays designed to measure CYTOKINES or signaling proteins, intracellular staining is required, but cell-surface staining is usually also performed in the same sample to allow phenotyping of the responding cell population. Depending upon the ANTIBODIES and EPITOPES targeted, this surface staining can sometimes be done together with the intracellular cytokine staining, if the targeted surface EPITOPES are resistant to the conditions of fixation and permeabilization [27,30]. If they are not, a surface staining step is carried out after activation but before fixation and permeabilization. In certain assays where proteins are transiently expressed on the cell surface (e.g., CD107, CD154), the ANTIBODIES to these markers can be added during the stimulation phase of the assay.

For most EPITOPES, surface and/or intracellular staining can be done by incubation with a cocktail of FLUOROCHROME-conjugated ANTIBODIES for 30–60 minutes at room temperature. Titration of ANTIBODIES is required for optimal staining (although many manufacturers offer pre-titered ANTIBODIES and cocktails of ANTIBODIES). The optimal titer for surface staining (unfixed cells) is often different than that for intracellular staining (fixed and permeabilized cells), even for the same cell-surface TARGET. This is due to increased non-specific binding of fixed and permeabilized cells.

As noted above, simple surface staining of whole blood can be done using a no-WASH ASSAY format. But more complex assays, such as intracellular cytokine staining, require washing, both after fixation and permeabilization steps, and after ANTIBODY staining.

Increasing automation and throughput

The relative complexity of sample preparation, especially for the more complex functional assays, has inhibited their application to studies of large numbers of animals or large clinical trials. However, robotic sample preparation devices are available and more are in development that would significantly aid in this process. For example, robotic workstations are commercially available that can aliquot whole blood into staining tubes, add ANTIBODIES, incubate, and add erythrocyte lysis buffer. There are also workstations that can perform cell washing, and thus can automate most of the steps of a washed assay. Integration of these robotic workstations can not only increase the number of samples handled in these applications, but can also lend considerable standardization to processes that otherwise are highly operator dependent.

Truly high-throughput sample processing, however, is best accomplished with multiwell plates. Both phenotypic and functional assays can be performed in 96-well plates. In fact, protocols for activation, processing, and analysis of samples for intracellular cytokine staining in a single 96-well plate have been published [18]. With the availability of plate-based loaders for flow cytometers, much larger numbers of samples can be processed in a single run, with minimal incremental technician time. Further automation of plate-based sample processing can also be accomplished using pre-formatted lyophilized reagent plates [31]. These have the advantage of long shelf life, allowing a single reagent batch to be used for a longitudinal study. They also tend to prevent errors of reagent addition that are otherwise difficult to prevent with complex multicolor experiments.

Data acquisition and analysis

Although sample preparation can be made parallel using multiwell plates and automation, sample acquisition is still a serial process. Fortunately, tube and plate loaders are available that can automate acquisition, and software can be used to annotate data files before they are run.

Data analysis involves the setting of "gates" or regions in one- or two-dimensional data space, followed by further analysis of the fluorescence properties of cells within those gates. Typically, viable cells of interest are first identified by their light scattering properties, using forward and SIDE SCATTER parameters. This may be followed by gating on subsets of LYMPHOCYTES, for example, $CD3^+$ (T cells), $CD19^+$ (B

cells), etc. Subsets of T cells ($CD4^+$, $CD8^+$) may be identified through further gating. In the case of functional assays, a responsive population, e.g., cytokine positive, is quantitated from the subset of interest, e.g., $CD8^+$ T cells.

The PRECISE placement of fluorescence and light scatter gates is historically done by eye, based upon recognition of typical patterns of staining (negative, dim, bright, etc.). However, cluster-finding algorithms are available in some current FLOW CYTOMETRY software programs that allow these gates to be drawn in a semi-automated way, and to be responsive to changes from one data file to another [18]. Such changes might include slight differences in staining intensities in different donors, between different experimental runs, etc. Using such cluster-finding algorithms, a gating template can be constructed that can then process all the data of a given study without requiring manual gate modifications for different subsets of data files. Visual inspection of the gates as applied to each data file is, however, still highly recommended.

A final component of data analysis that is often overlooked is the incorporation of flow cytometric data into a database that may also link it with other types of measurements (patient clinical data, etc.). Fundamentally, this requires the ability to extract measurements of interest from the FLOW CYTOMETRY data files into a spreadsheet along with any annotation associated with the data files. This can be done with modern FLOW CYTOMETRY software packages, such that manual entry of data into a spreadsheet is not necessary. A new data standard has been adopted by the flow community (FCS3.0 [32]), whose primary intent is harmonization and modernization, enabling current database technologies to manage both the experimental annotation, which may include read/write interfaces, and experimental measurements, which are read-only. Proprietary and open source tools are developing, so that enterprise-level flow experiments, covering years and many clinical sites can be managed more successfully. Such tools include data NORMALIZATION, sample quality assurance metrics and filters, pattern recognition algorithms and linkages with annotation information to enable data visualization and trend analyses (see for example, www.FICCS.org).

Examples and their application

Immunophenotyping assays

An example of an IMMUNOPHENOTYPING assay to quantitate T cell subsets is shown in Figure 3. Note that this was done as a "no-wash" assay in four colors, along with fluorescent beads to allow absolute counting. Fifty microliters of whole blood were stained with ANTIBODIES to CD45, CD3, CD4, and CD8. The blood was subjected to fixation/erythrocyte lysis, then run on a four-color flow cytometer. An ACQUISITION THRESHOLD was set on CD45 fluorescence to identify LEUKOCYTES. All cells above this threshold were collected, with acquisition halted at 20 000 $CD45^+$ events.

Analysis of this assay was done as follows. An initial gate was set on $CD3^+$ cells to identify all T cells. $CD4^+$ and $CD8^+$ T cells were then quantitated from a plot gated on $CD3^+$ cells. Note that this sample also contained counting beads, which are identified by very high fluorescence in all colors. Because a known number of these beads were added to a known volume of the blood sample, a simple calculation can convert the percentages of each cell subpopulation to an absolute count of cells per microliter of blood. Since percentages are relative to other populations, absolute counts have become a standard readout for reporting these types of results. Commercial software packages can perform these calculations in an automated fashion for this particular application.

Functional assays

An example of a functional assay (identifying intracellular cytokine production) is shown in Figure 4 [33]. This assay was performed by stimulation of whole blood with peptides derived from human cytomegalovirus (HCMV), a common herpesvirus that causes chronic, latent infection of various tissues. HCMV causes non-pathological infection in immunocompetent hosts, but is an opportunistic pathogen in immunocompromised hosts [34].

The peptides used for stimulation in this assay were a mixture of 138 15-mers, overlapping by 11 amino acid residues each, and spanning the

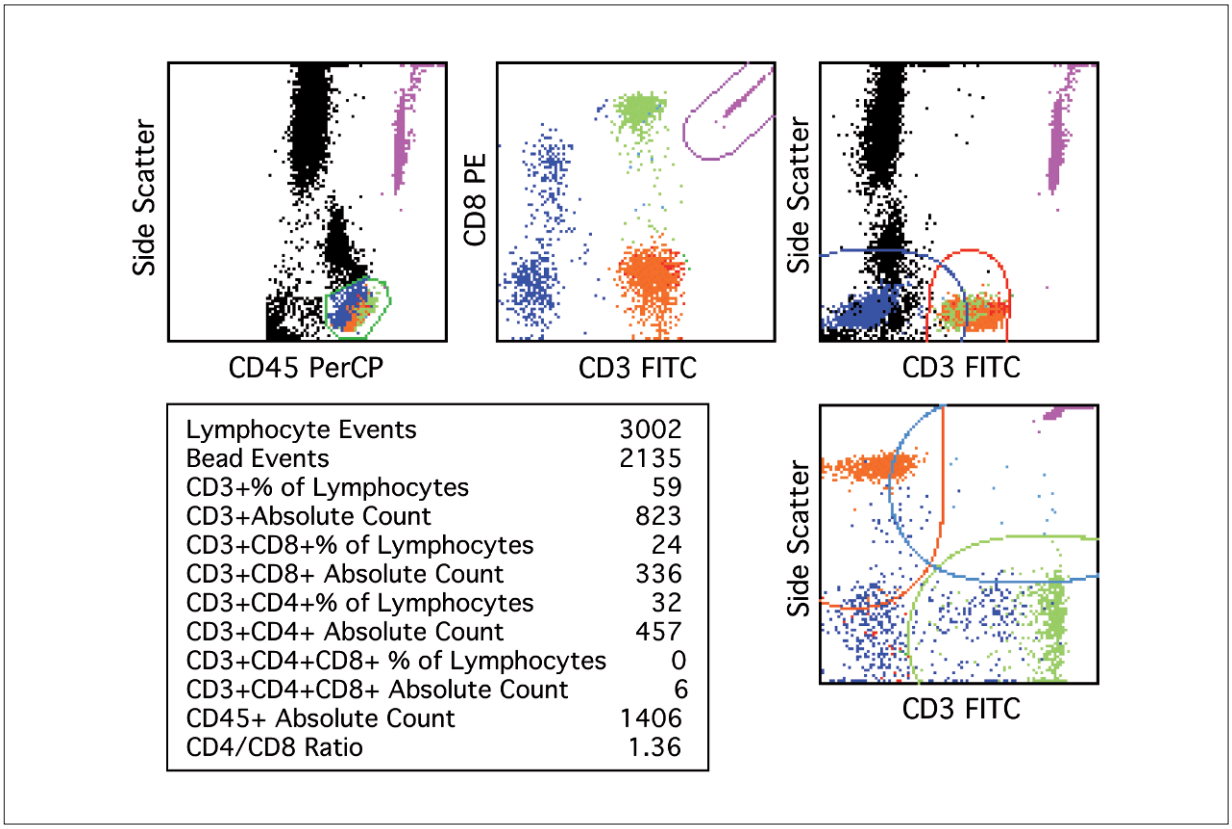


Figure 3. CD4 T cell enumeration assay from whole blood

Cells and counting beads were successively gated as described in the text, and the percentages and absolute counts of various lymphocyte subsets were automatically reported by the analysis software.

sequence of the pp65 glycoprotein of HCMV. pp65 is an immunodominant protein containing epitopes that stimulate CD4 and CD8 T cell responses in a variety of HLA haplotypes [35]. Using such overlapping peptides, both CD4 and CD8 T cell responses can be stimulated with relative efficiency [36]. The blood was stimulated with this peptide mixture for 6 hours in the presence of brefeldin A, followed by EDTA treatment, surface staining, fixation/erythrocyte lysis, permeabilization, and intracellular staining. The antibodies used included anti-IFN-γ, anti-IL-2, CD3, CD4, CD8, CD27, CD28, and CD45RA. The latter three markers identify memory and effector subsets of $CD4^+$ and $CD8^+$ T cells. A threshold was set on CD3 to allow collection of only $CD3^+$ cells, reducing the file size. Acquisition was stopped at 100 000 $CD3^+$ cells.

Analysis of this assay is outlined in the figure, and includes "snap-to" gates on major cell subsets [18]. These gates are able to move according to small changes in the staining pattern from one sample to the next. Cytokine-positive gates are rectangles that are "tethered" to the negative population, so that they shift in response to changes in the background staining. In this way, even very rare populations of cytokine-positive cells can be quantitated, whether they form a recognizable cluster or not.

If desired, one could combine the information from the above two assays to express the HCMV pp65-responsive cell populations as absolute num-

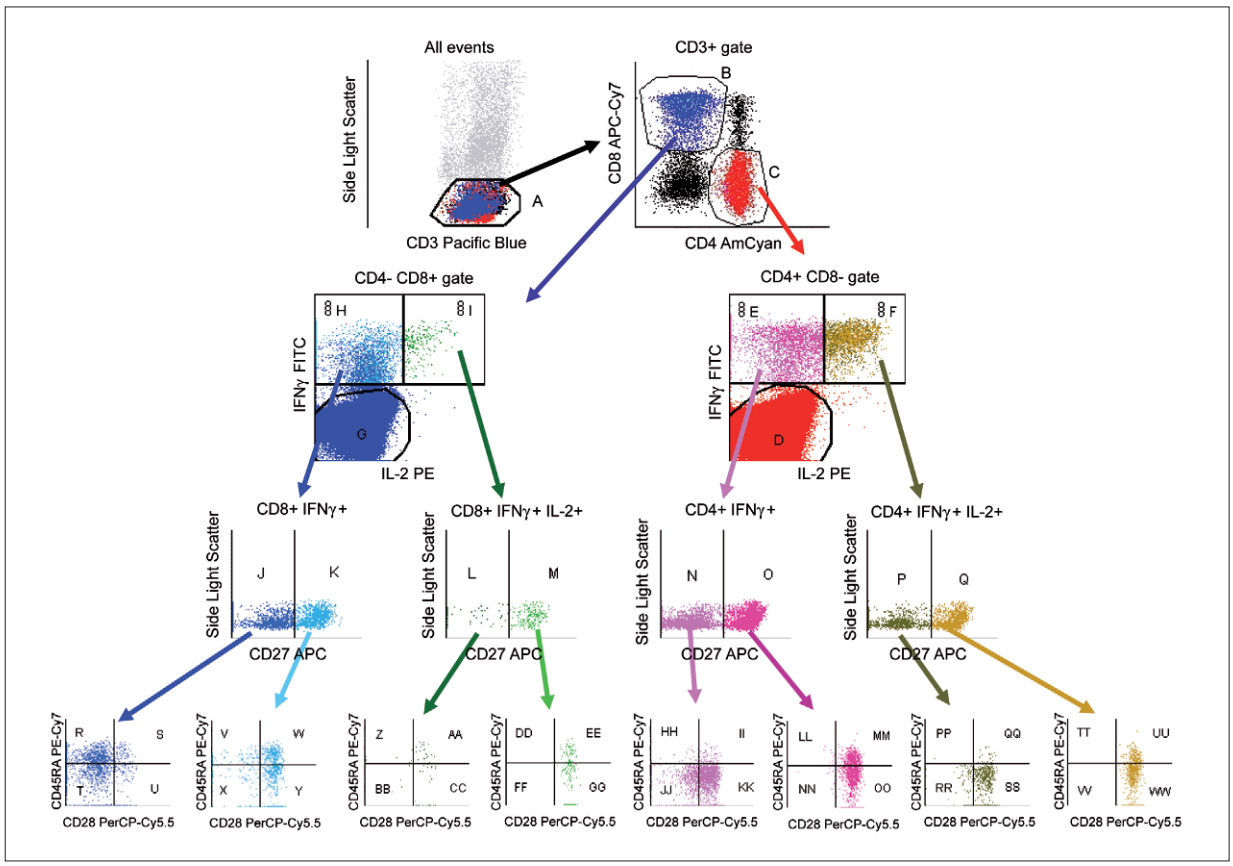


FIGURE 4. EIGHT-COLOR INTRACELLULAR CYTOKINE STAINING [33]

Peripheral blood mononuclear cells were isolated from whole blood, stimulated with a peptide pool from human cytomegalovirus, and stained with antibodies to eight markers that include two cytokines (IFN-γ and IL-2), T cell identification and subset markers (CD3, CD4, and CD8), and differentiation markers (CD27, CD28, CD45RA) that identify various forms of memory and effector T cells. The scheme for gating and identification of populations of interest is shown, and described further in the text.

bers of cells per microliter (or milliliter). This might provide more standardized results in populations that have varying numbers of CD4 and CD8 T cells (such as HIV patients).

What is the usefulness of identifying and quantitating intracellular cytokine responses? One major application is the development of new vaccines that are designed to elicit cellular immunity [37, 38]. These include both prophylactic and therapeutic vaccines for HIV, cancer, and certain other viral and intracellular bacterial pathogens. Establishing biomarkers of immunogenicity of vaccines (and eventually SURROGATE markers of protection) would greatly facilitate the comparison of different vaccine constructs and strategies, and allow more rapid improvement of vaccines for these diseases.

In the area of immunotoxicology, quantification of functional T cell responses, whether to specific ANTIGENS or to mitogens, could also provide valuable information [37]. While IMMUNOPHENOTYPING can potentially uncover gross changes in cellular subsets in response to a drug, functional assays can

uncover much more subtle changes. Suppression (or augmentation) of antigen or MITOGEN responses could give important clues to immunological side effects of drugs. This could be done as part of the early screening of drug candidates, before expensive animal studies are undertaken. Functional FLOW CYTOMETRY assays could also be used to monitor the dosing of immunomodulatory drugs in settings such as transplantation and AUTOIMMUNITY [39, 40]. By standardizing, automating, and increasing the ease and throughput of these assays, they can become ever more potent tools for immunological research, immunotoxicology, and clinical medicine.

Summary

FLOW CYTOMETRY is a powerful technique for analyzing cells in suspension, and has been extensively applied to the analysis of LYMPHOCYTES and their subsets. Flow cytometric assays can be divided into IMMUNOPHENOTYPING assays and functional assays, the latter requiring *in vitro* stimulation of cells in order to read out a response, such as phosphorylation or cytokine production. Such assays are being applied to the monitoring of clinical disease states and responses to vaccination or IMMUNOMODULATION, and they also have great potential for use in measuring immunotoxicology.

Suggested readings and website

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Robinson JP (ed) (2005) *Current Protocols in Cytometry*. Wiley, New York

Purdue cytometry bulletin board: *http://www.cyto.purdue.edu/hmarchiv/cytomail.htm*

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Microarrays

Stefan Wild, Ute Bissels, Barbara Schaffrath and Andreas Bosio

Introduction

The phenotype of a cell is determined by the amount, the proportion and the condition of proteins present in this cell. Although every cell in an organism possesses the same genetic information, only certain genes are transcribed into MESSENGER RIBONUCLEIC ACID (mRNA) according to the function and demands of the cell. Based on the information provided by the mRNA, the information is translated into the corresponding protein, contributing to a distinctive set of proteins for every cell and every status of the cell, defining its phenotype. The mapping of the whole human genome was completed in 2004 [1]. Researchers are focusing now on the illumination of functions and interactions of genes and gene products by measuring, for example, the number of activated genes. DNA MICROARRAY technology, as well as other established DNA and RNA detection methods, utilise the characteristic of RNA strands to form helices due to complementary sequences. This process of combining two RNA strands to form a double helix is called HYBRIDISATION. Since Southern introduced the blotting technique [2] for DNA, the HYBRIDISATION process has been used in a wide range of techniques for the recognition and quantification of DNA or RNA. Such "classical" HYBRIDISATION techniques measure one DNA or RNA sequence per HYBRIDISATION using a specific probe. In contrast, DNA microarrays consist of several thousands of specific probes arrayed in a two-dimensional pattern allowing the parallel investigation of thousands of genes.

Principle of the technology

Microarrays are miniaturised devices made for the analysis of targets of interest with a high degree of parallelisation. Initially, the technology evolved around the analysis of mRNA levels in cells in different states, taking "classical" HYBRIDISATION-based technologies to a new level. For "classical" HYBRIDISATION-based analysis, genomic DNA (Southern) or RNA (Northern), extracted from the tissue of interest, is immobilised on a membrane. A single specific nucleotide sequence (the probe) that is complementary to the sequence of interest, is labelled and applied to the membrane to subsequently detect the corresponding gene or gene transcript (Fig. 1). For array analysis, this principle is reversed and applied to thousands of sequences of interest by immobilising DNA fragments (probes) with distinct sequences on a SUBSTRATE (a membrane, glass, silicon, or plastic slides) at defined positions (see Box 1). Nucleic acids from the cells of interest are labelled, applied to the SUBSTRATE for HYBRIDISATION and the hybridised nucleic acids are identified by their position on the array.

The workflow of this process is illustrated by means of a DNA MICROARRAY experiment: In a typical scenario, GENE EXPRESSION of tumour cells, for instance, is compared to that in normal cells. RNA from tumour and normal cells is extracted from the respective tissue (Fig. 2). The RNA is transcribed into its reverse complementary copy, the so-called cDNA. The cDNA derived from tumour cells and normal cells is labelled and applied to the DNA array. During the HYBRIDISATION step, the labelled nucleic acids bind to the complementary sequences of the respective probes. After washing away all unbound labelled nucleic acids, the signal intensities for each probe position are determined. After signal intensi-

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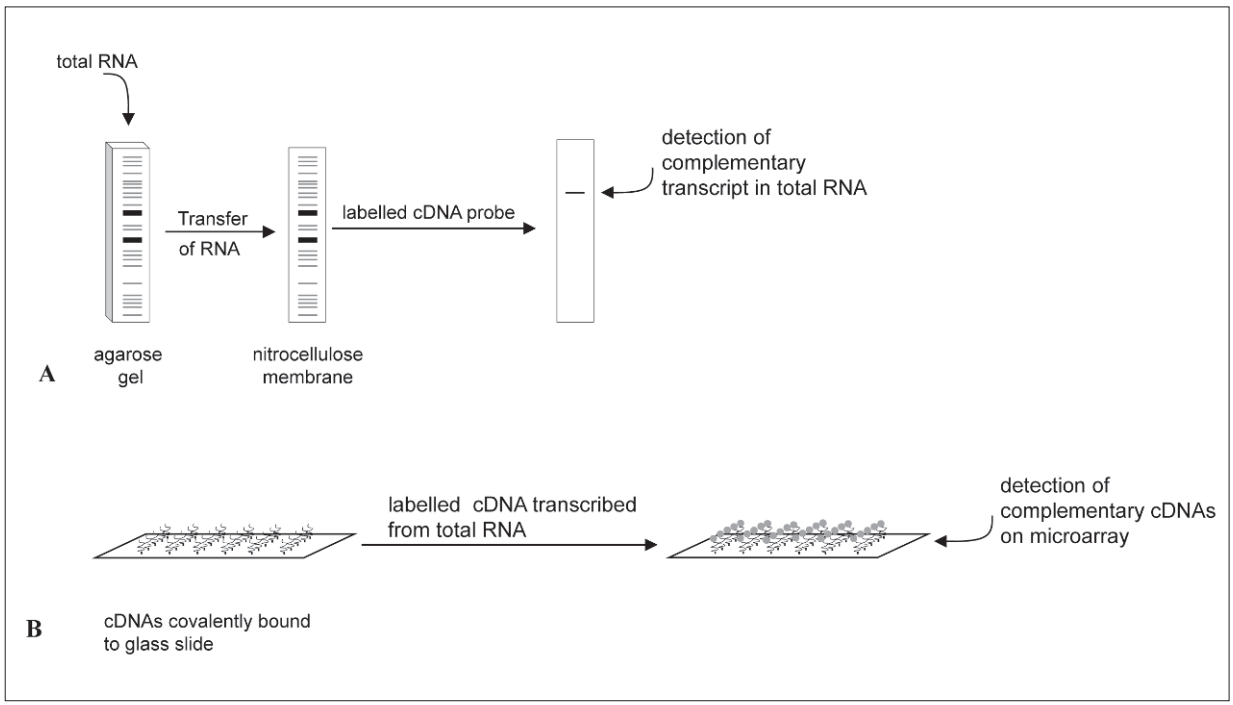


Figure 1. Comparison of traditional Northern-blot and DNA microarray

A: Total RNA of the tissue of interest is separated by gel electrophoresis and is blotted to a membrane. A labelled cDNA probe complementary to the transcript of interest is labelled and hybridised to the membrane. If the transcript is present in the total RNA, a signal can be detected due to hybridisation of probe and transcript. One experiment – one gene using a single labelled probe.

B: Several cDNAs (hundreds to thousands) complementary to mRNA transcripts of selected genes are covalently bound to a glass slide at defined positions (spots). Total RNA from the tissue of interest is transcribed into cDNA and labelled by reverse transcription. The labelled cDNA is hybridised to the bound cDNAs. Signals can be detected after hybridisation of two complementary cDNAs.

ties have been generated for all probes on the array, signals derived from normal cells and tumour cells are compared and differences in GENE EXPRESSION are identified. The altered expression of certain genes in the tumour, such as oncogenes, can help to typify the tumour. Combining the expression profile with clinical data may then be used to decide on the prognosis and the best therapy for the patient.

In addition to the described GENE EXPRESSION PROFILING, microarrays are also used to investigate other nucleic acids like genomic DNA [3] or non-coding RNAs [4] including MICRORNAS (miRNAs) [5–9]. In addition, the array principle has also been adapted to other ANALYTES such as proteins [10] or carbohydrates [11].

Due to the parallel measurement of up to thousands of ANALYTES, microarrays offer the opportunity to observe complex biological systems while using minimal amounts of sample material. Although in the following sections specifications and workflow procedures are mainly related to DNA microarrays for GENE EXPRESSION PROFILING, the general aspects hold true for other MICROARRAY-based technologies as well.

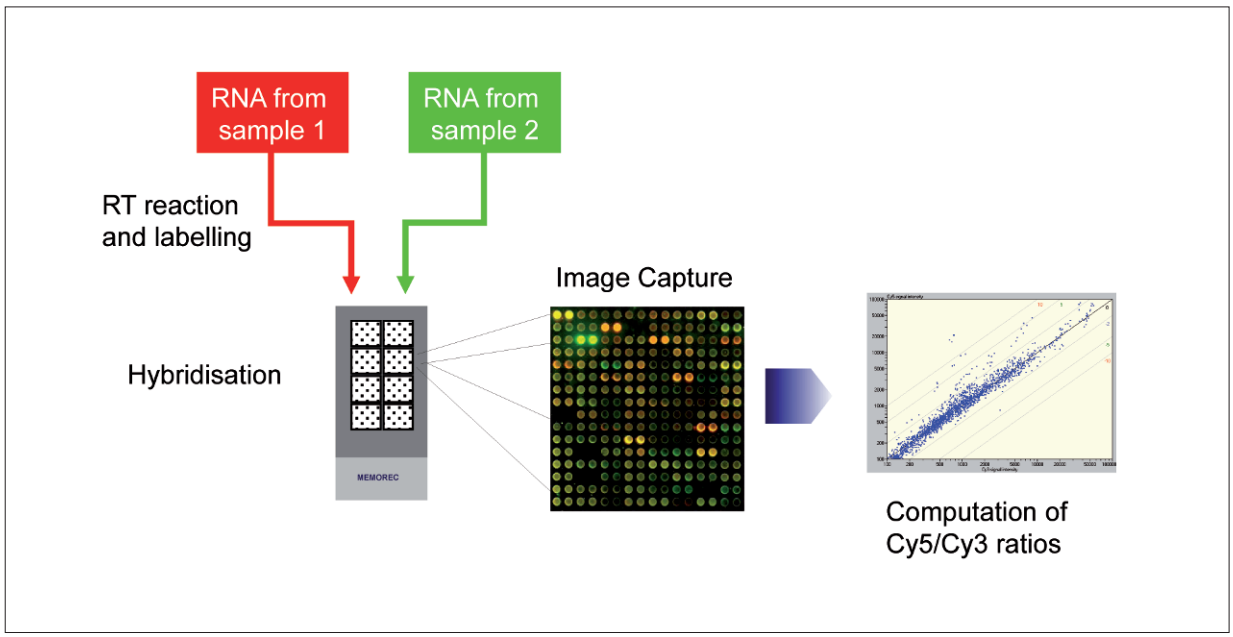


FIGURE 2. WORK FLOW DIAGRAM OF MICROARRAY ANALYSIS

BOX 1. PRODUCTION OF MICROARRAYS

A variety of different array substrates (membranes, plastics, glass), in combination with a range of different coatings, are used as the solid phase for microarray production. Coatings permit the functionalisation of substrates with reactive groups, like aldehyde, epoxy, or isothiocyanate moieties, to bind DNA probes on the substrates.

The DNA probes can be directly synthesised on the microarray substrate (*in situ* synthesis) or the complete DNA probes are spotted on the substrate. The *in situ* synthesis by photomediated synthesis or ink-jet technology, allows a parallel production of OLIGONUCLEOTIDE ARRAYS, comprising oligonucleotides of 20–60 nucleotides in length [31, 32]. The use of short oligonucleotides (20–30 base pairs) is suitable to differentiate between perfectly matched duplexes and single-base or two-base mismatches [33–35]. When working with short oligonucleotide probes, the use of several different oligonucleotides corresponding to a single gene is typically required to enhance the reliability of the hybridisation signals [36].

Alternatively, cDNA fragments or pre-synthesised oligonucleotides with a length of up to 70 base pairs are spotted on the functionalised substrate in two manners: contact printing and non-contact printing.

CONTACT PRINTING typically involves rigid pins dipping into the spotting buffer containing the DNA probes. The drop at the tip of the pin is brought close to the surface at a given position and a tiny drop remains on the surface. Non-contact printing methods are based on ink-jet technology. The spotting buffer containing the DNA probes is dispensed as tiny droplets from the print head. Independent of the spotting mode, binding of the DNA probes occurs at the position of the drop. After the actual spotting process is completed, unbound DNA is removed and the reactive substrate is blocked to avoid non-specific (independent of the provided sequence) binding of nucleic acids during hybridisation. The microarrays are now ready for processing.

Application of microarrays

Preparation and quality of RNA

The first crucial step to achieve reliable GENE EXPRESSION results is RNA isolation. RNA is susceptible to chemical hydrolysis and to RNases, widespread enzymes that digest RNA molecules into small pieces. If the RNA is slightly degraded or contaminated by residual genomic DNA, for instance, the results may be biased and irreproducible (see also Box 2). Commonly, RNA is extracted from cells or tissues using organic solvents or silica filter-based methods. Since RNA extraction protocols may influence the outcome of the expression analysis, the same extraction procedure should be used for all samples analysed in one set of experiments.

Amplification of RNA

The SENSITIVITY of MICROARRAY experiments strongly depends on the amount of material used for HYBRIDISATION. As the amount of RNA is usually limited, different AMPLIFICATION methods are available. The most common method utilises T7 DNA-dependent RNA polymerase to amplify RNA. The mRNA is first reverse transcribed to cDNA. The primer used for the reverse transcription additionally comprises the sequence of the T7 promotor. After the second strand synthesis, the T7 promotor is used by the T7 DNA-dependent RNA polymerase for *in vitro* transcription. The T7 DNA-dependent RNA polymerase repeatedly transcribes the same cDNA thereby amplifying the original RNA (Fig. 3) [12]. In case even higher SENSITIVITY is needed, the amplified RNA can again be used as SUBSTRATE for cDNA synthesis and a second round of T7-based AMPLIFICATION. Alternatively, a variety of other AMPLIFICATION methods like PCR-based AMPLIFICATION methods have been developed (Fig. 4) [13]. Due to the slightly different properties of the different RNAs, such as length, sequence or GC content, the AMPLIFICATION efficiency can vary for different RNAs, again depending on the AMPLIFICATION method. Therefore, to allow comparison of different RNA samples, it is advisable to use the same AMPLIFICATION method for all samples. The most sensitive AMPLIFICATION methods allow MICROARRAY experiments from as little as a single cell (see also section on "Global RNA AMPLIFICATION and MICROARRAY analysis of T cell subpopulations" and Fig. 4).

Dyes, labelling and hybridisation methods

Most commonly, fluorescent dyes are used to detect the hybridised samples on microarrays, but alterna-

Box 2. Quality of total RNA

Integrity and purity are the most critical factors for the quality of RNA.

- Ratio of 28S rRNA and 18S rRNA should be 2, reflecting the higher molar mass of 28S rRNA compared to 18S rRNA. A more precise quality measure is given by the RNA integrity number (RIN) calculated by the Agilent Bioanalyser.
- Ratio of the extinction 260 nm/280 nm should be between 1.8 and 2.0.
- The sample can be treated with RNase-free DNase to avoid contamination of genomic DNA.
- Protocols for RNA extraction have to be adapted according to the analysed tissue (e.g. high fat content or fibrous tissue)
- The choice of the preparation protocol may have an influence on the range of transcript lengths present in the extracted RNA (e.g. silica filters usually have a cut-off size of about 50–100 bases. Therefore, preparations derived in this way do not contain the whole range of fragment lengths. This might have an impact on the subsequent steps (labelling, amplification, or hybridisation).

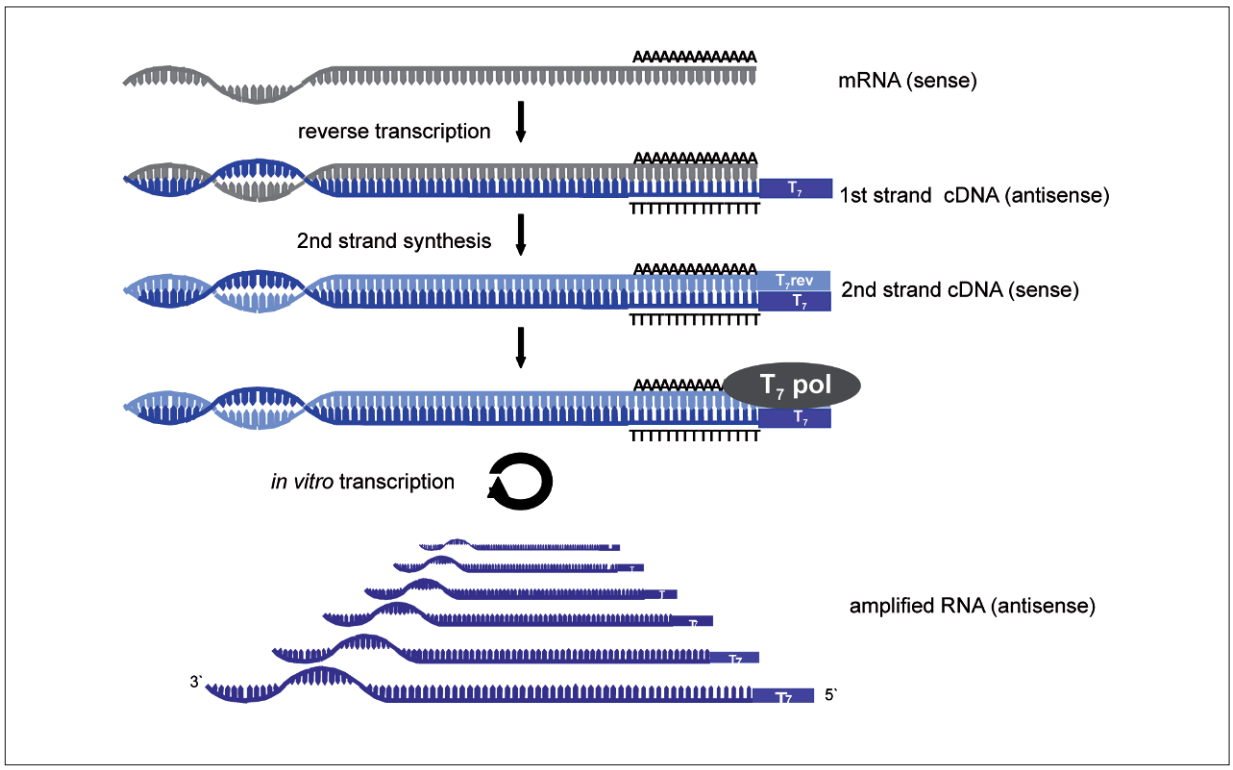


FIGURE 3. SCHEMATIC DIAGRAM OF T7 POLYMERASE-BASED MRNA AMPLIFICATION

tive labelling methods using radioactivity or silver particles, for example, can also be applied.

In DIRECT LABELLING protocols, the labelled nucleotides are incorporated during the cDNA synthesis or the T7 DNA-dependent RNA polymerase-based AMPLIFICATION. Since the incorporation rate of labelled nucleotides is compromised by the partly bulky fluorescent dye, two-step labelling protocols (INDIRECT LABELLING) have also been established. During a two-step labelling procedure, nucleotides labelled with a small molecule like biotin or an aliphatic amine, are incorporated by the polymerase. In a second step, the fluorescent dyes are linked to the modified nucleotides *via* streptavidin or amine reactive groups like NHS esters. Depending on the system, the second step of the labelling protocol can also be performed after the HYBRIDISATION step (on-chip labelling).

After the labelling, the samples are hybridised on the MICROARRAY. The HYBRIDISATION can either be achieved by simple diffusion of the TARGET DNA molecules to the corresponding probes, or probe TARGET interaction can be assisted by moving the HYBRIDISATION mixture on top of the array. After the HYBRIDISATION step has been completed, unbound labelled TARGET molecules are removed by washing the array. Finally, the array is dried.

To minimise experimental variance caused by some of the processing steps, like the labelling or HYBRIDISATION, it is advisable to perform replicate MICROARRAY experiments using the same sample.

The HYBRIDISATION is usually performed as a one- or two-colour experiment. For one-colour experiments, each sample is hybridised on one array and the signal intensities derived from different arrays are compared. When using two colours, the two samples to be compared are labelled with different dyes and hybridised together on the same array. The direct comparison of the two samples on one array

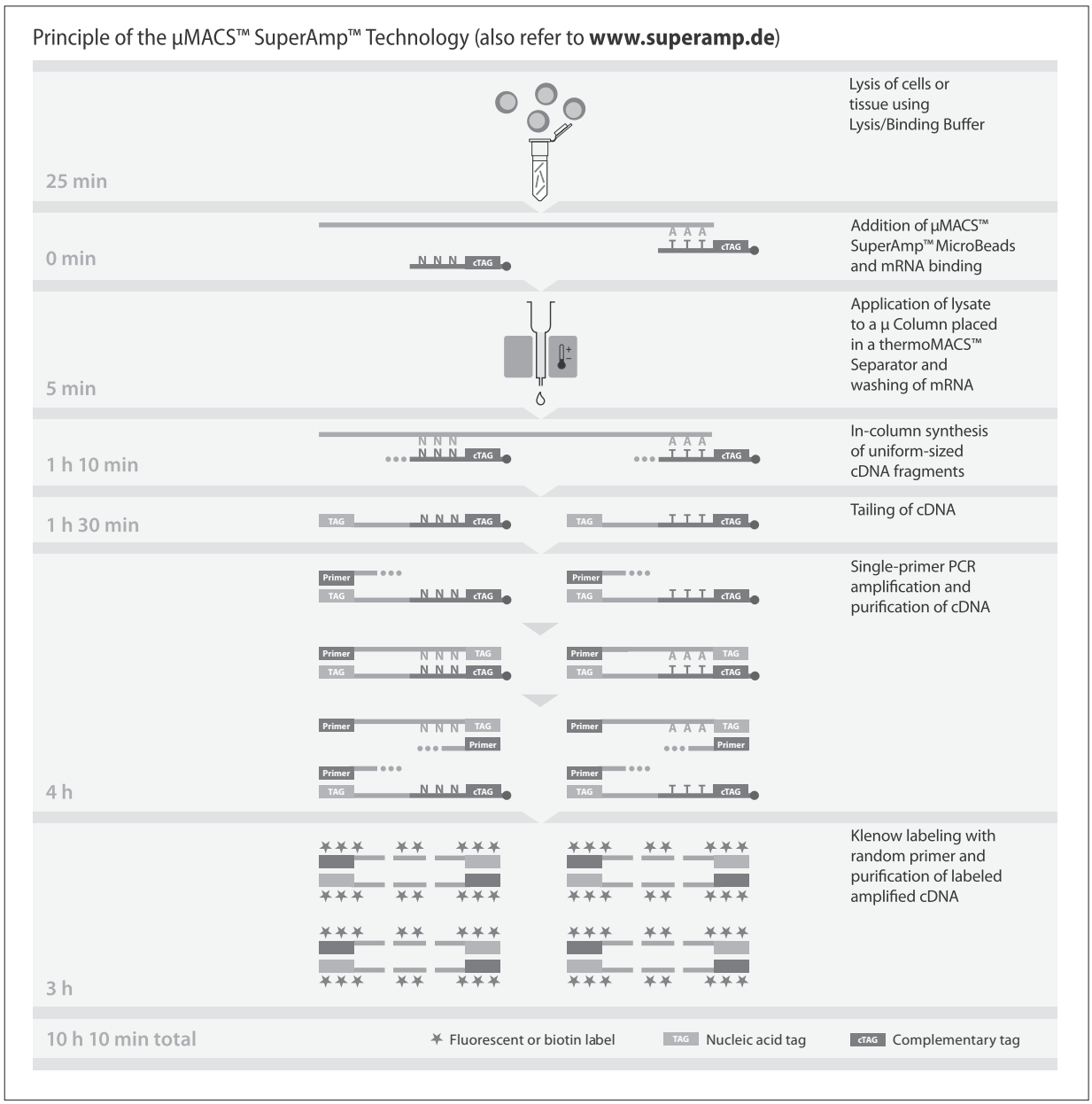


FIGURE 4. SCHEMATIC DIAGRAM OF A GLOBAL PCR-BASED mRNA AMPLIFICATION

has the advantage that any experimental bias related to the array or the HYBRIDISATION step will affect both samples, therefore reducing detection of artefacts. When working with fluorescent dyes, however, the integrated dyes not only differ in their emission wavelength, but also in the fluorescence intensity gained per dye, due to wavelength-dependent scanner properties, diverse quantum yields of the dyes,

Box 3. Normalisation of microarray data

Integrity and purity are the most critical factors for the quality of RNA.

The main idea of NORMALISATION for dual-labelled samples is to adjust differences in the intensity of the two labels. Such differences result from the efficiency of dye integration, differences in amount of sample and label used, and settings of laser power and photo-multiplier. NORMALISATION of one channel arrays mainly corrects spatial heterogeneity. Although NORMALISATION alone cannot control all systematic variations, NORMALISATION plays an important role in the earlier stage of microarray data analysis because expression data can vary significantly due to different NORMALISATION procedures. A number of NORMALISATION methods have been proposed, but there is no general rule which method performs best. The NORMALISATION method strongly depends on several factors like the number of detectable genes, the number of regulated genes, signal intensities, quality of the hybridisation, etc.

For a rough classification, global NORMALISATION can be distinguished from local (signal intensity-dependent) NORMALISATION and NORMALISATION via transcripts known to be non-regulated or spike-in controls.

If global NORMALISATION is used, a single NORMALISATION factor is applied to all detectable genes, leading to a linear shift of all signal intensities. The underlying assumption is that constant systematic variations occur, including a lower integration rate of one dye in respect to the second dye. However, global NORMALISATION based on the median of all detected genes can only be used if a sufficient number of genes are non-regulated. If it is expected that most of the genes are regulated (which is of special interest regarding miRNA arrays) a set of "housekeeping genes" or spike-in controls should be included in the array configuration. Because housekeeping genes (by definition) are not regulated, the signal intensities of those genes should be the same on dual-labelled arrays. Using local NORMALISATION, a different NORMALISATION factor is calculated for every gene. Local NORMALISATION offers the opportunity of a signal intensity-dependent NORMALISATION. Some variations (e.g. laser settings) have different impacts on detected genes depending on their signal intensity. Thus, a non-linear shift of the signal intensities can be achieved based on the signal intensity of each single spot.

In the field of miRNA microarray research, NORMALISATION *via* spike-in controls is preferably used, as global NORMALISATION methods may fail due to (a) missing housekeeping miRNA, (b) limited number of expressed miRNAs and (c) a general up- or down-regulation of many miRNAs. The used spike-ins represent a set of synthetic RNAs, which have no similarity to any known miRNA. The spike-ins are added to all experimental and control samples, and all signal intensities of the investigated samples are normalised using the median of the spike-ins.

or different stabilities of the dyes. Therefore, the raw data gained by two-colour MICROARRAY experiments has to be corrected for such dye effects. The methods used to centre or normalise the signal intensities for both wavelengths are based on the assumption that some of the genes, like housekeeping genes, are not regulated (see Box 3). The differences found for these genes can therefore be used to calculate a factor reflecting the different dye properties. As the reproducibility of array production and MICROARRAY HYBRIDISATIONS has dramatically improved, there is a trend in favour of single-colour HYBRIDISATIONS.

Control samples

The measurement of GENE EXPRESSION in a given sample is usually referred to the GENE EXPRESSION in other samples, here referred to as "control". Obviously, it is very important to choose the right control in order to gain valuable data. The best controls in most experiments are untreated cells or unaffected tissue of the same origin as treated cells or affected tissue, respectively. However, for practical or ethical reasons, it is not always possible to receive untreated cells or healthy tissue of the same origin, which is especially true for material derived from patients. If it is impos-

sible to get matched control samples, a "related" control can be established, for instance, by pooling RNA from different individuals to reduce the effects of particular properties of single individuals in the control. In some cases, cell lines might also be a sufficient control. Alternatively, a pool of all samples used in an experimental series can work as the control (see Box 4). However, a sample pool carries the risk of missing genes that are consistently expressed differentially in all samples. In general, controls should either be case-matched to the samples of interest or consist of pooled material to compensate for individual differences.

Array data: acquisition, analysis and mining

Data acquisition

Data acquisition of MICROARRAY experiments consists of two parts: the read-out of the MICROARRAY, meaning the detection of the signals, and the following image analysis. Whereas films have been used to detect radioactive signals, nowadays predominantly MICROARRAY scanners are used to excite commonly used dyes and measure the emitted fluorescence signals. The picture derived from the read-out of the MICROARRAY is saved as greyscale tiff images for further analysis. During the next step, the signal intensity of each spot is determined and assigned to the gene represented by the given spot using appropriate image analysis software. In addition, the background signal, usually gained from the surrounding area of each spot, is subtracted from the signal to receive the net signal intensity. Spots of poor quality (empty or negative spots, irregular shape, spots showing background smears) can be excluded from further analyses. The set of data that results from the data acquisition step is referred to as primary data.

Data analysis and mining

For the analysis of the primary data, weak signals are excluded as non-reliable. The minimum reliable signal intensity of a spot can be determined by setting a minimum threshold for signal intensities, which is either dependent on the background or on nega-

Box 4. The reference strategy for two-colour hybridisations

In microarray experiments, the direct comparison of absolute signal intensities of different microarrays can be critical due to different hybridisation efficiencies. To avoid this obstacle, two-colour microarray hybridisations can be performed. In two-colour microarray hybridisations, the sample, labelled with Cy5, for instance, and the control, labelled with Cy3, are hybridised on the same microarray. As the labelled molecules compete for the same probes on the microarray, the hybridisation efficiency is also the same, and allows a direct comparison of sample *versus* control. Therefore, the ratio of the signal intensities of the two dyes represents the proportion of the analyte in the sample compared to the control.
The principle of two-colour hybridisation can be extended to compare more than two samples by applying a reference scheme. For a microarray reference experiment, each of several samples and controls are hybridised *versus* the reference. The reference can then be used to compensate differences of the hybridisation efficiency for each microarray and allows standardisation and cross-referencing of microarray experiments. For the analysis of mRNA expression profiles, references consisting of total RNA mixtures are used [37]. For miRNA analysis, universal references consisting of known amounts of synthetic miRNAs are available [29]. Besides the cross-referencing of array experiments, such a reference allows the absolute quantification of miRNAs. The Universal Reference, consisting of an equimolar pool of about 1000 miRNAs, is labelled and hybridised versus each sample in a two-colour microarray approach. In this way, each single miRNA is quantified in comparison to an identical standard, compensating the bias related to sequence, labelling, hybridisation or signal detection.

tive controls. For some microarrays, *p* values giving an estimate of the likelihood of the signal differing from background signals are used to indicate the reliability of the detected genes. To compare different samples, ratios of the signal intensities gained, such as for sample *versus* control, are computed for every detected gene. To correct for different labelling and HYBRIDISATION efficiencies, as well as for potential dye bias in two-colour MICROARRAY hybridisations, the signal intensities are centred or normalised prior to calculating the ratios (see Box 3).

Because of the multiparametric nature of MICROARRAY experiments, data mining and bioinformatics analysis are essential for interpretation of the numerical data produced by (series of) MICROARRAY experiments. Starting from relatively simple demands for appropriate visualisation of the data, bioinformatics tools are necessary to focus on candidate genes and reveal subtle changes in expression patterns.

A reliable identification of candidate genes by statistical methods is only possible if a sufficient number of replicate experiments have been performed. Technical replicates using the same starting material are usually performed to define the overall reproducibility of MICROARRAY experiments. Biological replicates are important to discriminate individual differences (e.g. patient specific) from general changes of GENE EXPRESSION (e.g. disease specific).

Additional bioinformatics methods can be used to identify groups of genes showing a comparable regulation. One method commonly used is the HIERARCHICAL CLUSTER ANALYSIS where genes and arrays are ordered by similarity in expression [14]. Due to the overwhelming amount of data, it is often difficult to understand MICROARRAY results in the light of certain biological questions. To assist researchers in interpreting the results, MICROARRAY data can be combined with knowledge stored in diverse databases like pathway information, genomic localisation or protein family classification.

Different data analysis tools can be applied to identify genes that may be related to a disease or treatment of interest. Linking the data to biological knowledge can also elucidate possible functions of the genes interest. Succeeding experiments using mostly molecular biology techniques like RT-PCR, *in situ* HYBRIDISATION, RNAi, knockout experiments, etc., are commonly performed to validate and corroborate the biological function concluded from the MICROARRAY data.

Examples of microarray experiments

Global RNA amplification and microarray analysis of T cell subpopulations

Naïve T cells differentiate in response to pathogens into multiple $CD4^+$ and $CD8^+$ subsets. To improve the understanding of this differentiation process as well as the nature of the different subsets, GENE EXPRESSION PROFILING has been used. As an example, MICROARRAY experiments were performed from ten different subpopulations covering the major stages of post-thymic $CD4^+$ and $CD8^+$ T cell differentiation (Fig. 5) [15]. The $CD4^+$ and $CD8^+$ subsets were isolated by immunomagnetic and flow cytometric cell sorting (see chapter B3) based on the expression of CD4/CD8, CD27, CD28, CD45RA, and CCR7. These markers characterise the major steps of T cell differentiation from naïve to highly differentiated cells in humans [16, 17]. The GENE EXPRESSION profiles were generated from multiple T cell subsets independently gained from two blood samples. As only limited cell numbers can be isolated from 20 mL of blood, a global PCR AMPLIFICATION method was applied allowing MICROARRAY experiments from 1000 cells per T cell population.

For the AMPLIFICATION of RNA from small cell numbers, loss of material is critical and the pipetting of samples from one tube to another should be avoided as much as possible. For the global AMPLIFICATION, the cells were collected in a small volume of buffer and lysed (Fig. 4). Then, superparamagnetic oligo dT microbeads were directly added to the cell lysate binding the poly(A) residues of the mRNA. The labelled cell lysate was applied to a column that was placed in the magnetic field of a heatable permanent magnet. The magnetically labelled mRNA was retained in the strong magnetic field while effective washing steps removed all other cell components. In-column cDNA synthesis and purification was per-

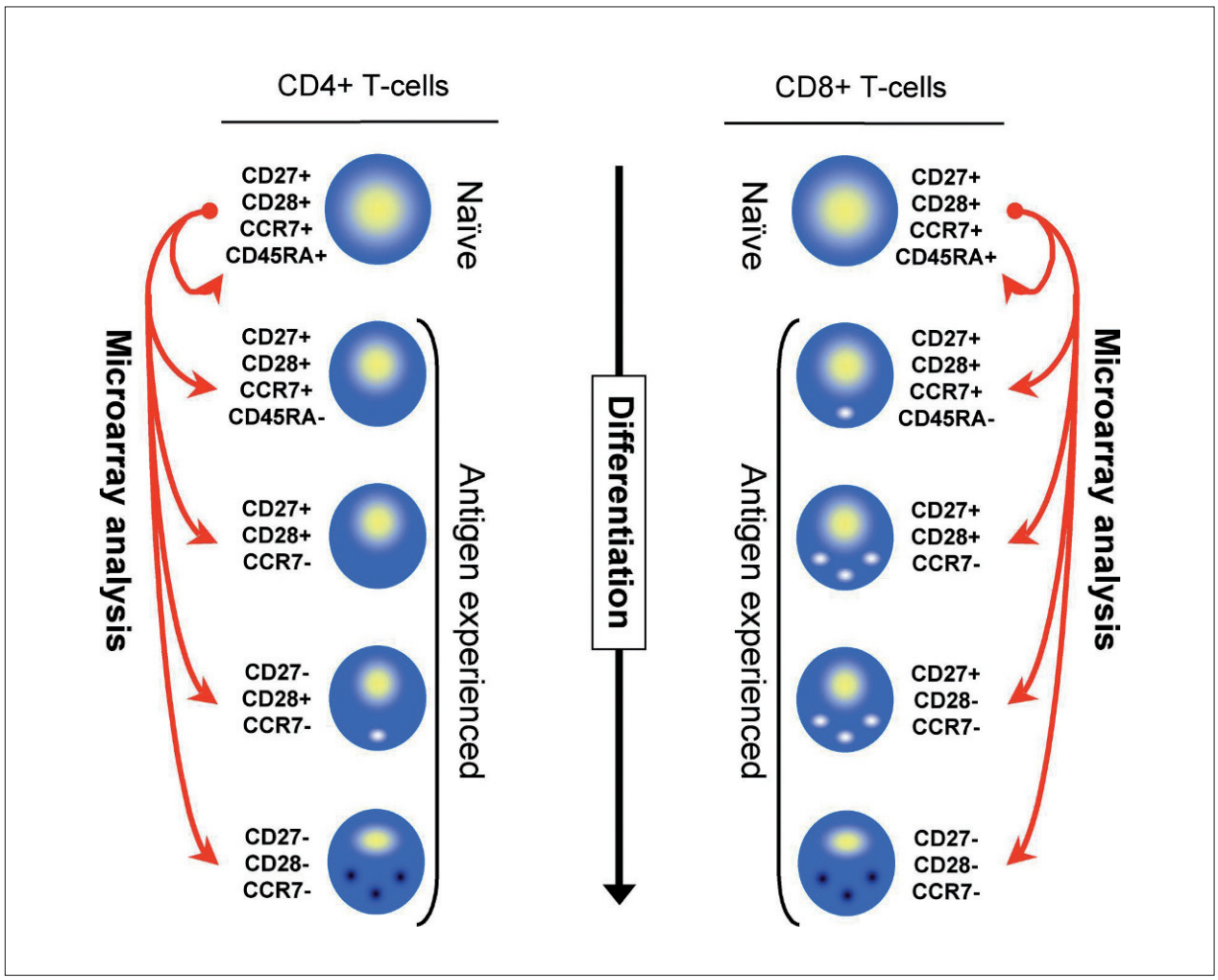


FIGURE 5. MICROARRAY ANALYSIS OF CD8⁺ AND CD4⁺ T CELL SUBPOPULATIONS DEFINING DISTINCT STAGES OF DIFFERENTIATION

formed in the same column used for mRNA isolation to avoid loss of material. Oligo dT as well as randomer oligonucleotides coupled to microbeads were used as primers for the cDNA synthesis. Thereby, cDNA fragments of uniform size were generated and each transcript was represented by several cDNA fragments enabling uniform AMPLIFICATION during PCR. After eluting the cDNA fragments from the column, a tag was added to the 3' end of each cDNA fragment by utilising a terminal deoxynucleotidyl transferase. A global PCR amplified the uniform-sized cDNA fragments 10^6-fold, resulting in sufficient TARGET material for MICROARRAY HYBRIDISATION. The PCR performed with a single primer enabled unbiased AMPLIFICATION due to the uniform annealing temperature. The primer binding site at the 3' end was added during cDNA tailing. The complementary sequence of the tag was inserted at the 5' end of the cDNA fragments during cDNA synthesis. After purification of the PCR products, a Klenow fragment labelling procedure with random primers in the presence of labelled nucleotides, in this case, Cy3-

dCTP or Cy5-dCTP, yielded labelled DNA fragments that were used for MICROARRAY HYBRIDISATION.

All differentiated T cell subsets were hybridised against the corresponding naïve T cells as control in two-colour MICROARRAY experiments. Therefore, the genes found differentially expressed on the microarrays represented potential genes related to the differentiation from naïve to antigen-experienced T cells.

For the first differentiation stage ($CD27^+/CD28^+/CCR7^+/CD45RA^+$), about 15% of the detected genes were found to be differentially expressed and this proportion increased for stages 2–5 to about 50%, which is consistent with the differentiation process.

A detailed analysis of the differentially detected genes revealed the acquisition of a cytolytic program by the highly differentiated T cells represented by the expression of genes encoding for the lytic granule membrane protein LAMP-3 and the CYTOTOXIC factors granzyme B and perforin. The up-regulation of these genes giving rise to lytic and CYTOTOXIC proteins supported the idea of CYTOTOXIC T cells as late differentiation state.

Another interesting set of genes was found down-regulated in highly differentiated T cells. These genes encode for proteins involved in cell cycle entry and/or cell proliferation, as well as anti-apoptotic factors, suggesting a quiescence state and limited survival potential for the highly differentiated T cells under stress or upon activation.

Overall, during the differentiation process, the changes in GENE EXPRESSION for the differentiated T cells compared to the naïve T cells became increasingly similar between $CD4^+$ and $CD8^+$ T cells. So despite the clear differences between naïve $CD4^+$ and $CD8^+$ T cells, the differentiation process might be orchestrated by analogous changes in the GENE EXPRESSION profile.

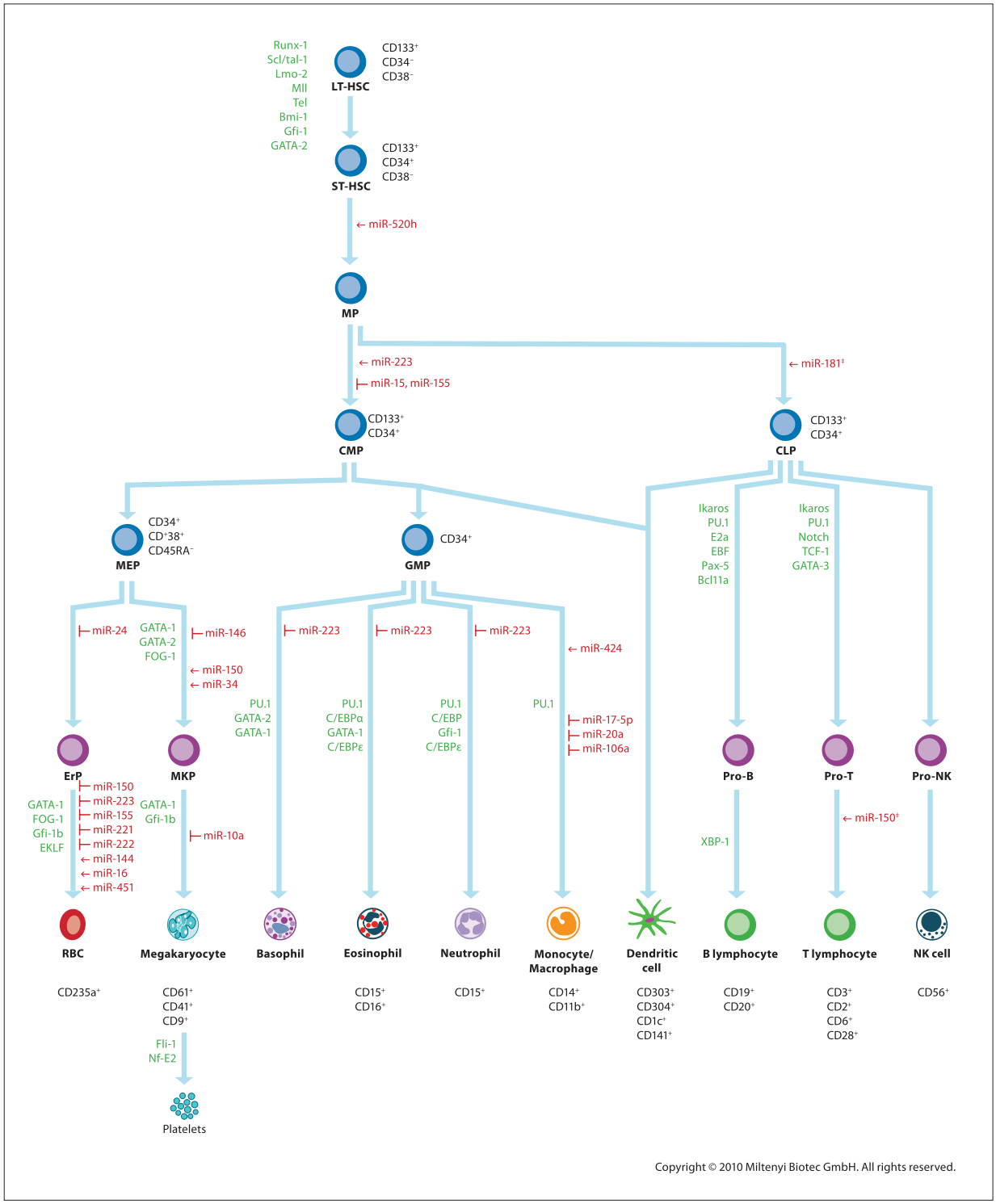
In summary, the GENE EXPRESSION analysis using global RNA AMPLIFICATION for MICROARRAY experiments suggested functional changes especially during the late differentiation state pointing to CYTOTOXIC potential and limited lifespan. In addition, common changes in the GENE EXPRESSION pattern pointed to a similar differentiation process for $CD4^+$ and $CD8^+$ T cells.

miRNA microarrays for analysis of miRNA expression in haematopoietic cells

miRNAs, short non-coding RNAs of 21–23 nucleotides (nt) in length, regulate TARGET mRNAs post-transcriptionally. A widely used method to analyse miRNAs are miRNA microarrays [5–9]. In contrast to classic mRNA expression profiling, the establishment of miRNA MICROARRAY platforms represents a greater challenge with regard to probe selection, labelling and handling. The physicochemical properties of miRNAs that are determined by their short length make it necessary to tightly adjust parameters such as the HYBRIDISATION temperature and HYBRIDISATION buffer. Taking these properties into account, different miRNA MICROARRAY platforms have been developed. They permit the study of the role of miRNAs in many different cellular, developmental, and physiological processes, such as haematopoietic lineage differentiation.

In 2004, it was shown for the first time that miRNAs are involved in haematopoietic lineage differentiation [18]. Further analysis in numerous research groups showed that miRNAs fine tune each step in HAEMATOPOIESIS (Fig. 6). It was demonstrated, for instance, that miR-150 drives megakaryocyte-erythrocyte progenitor (MEP) differentiation towards megakaryocytes at the expense of erythroid cells [19]. Erythropoiesis is promoted by miR-451, miR-16 and miR-144 and negatively regulated by miR-150, miR-155, miR-221 and miR-222 [20–23]. Furthermore, it was shown that the miRNA cluster miR-17-5p-92 controls monocytopoiesis [24] and that miR-424 is up-regulated during monocyte/macrophage differentiation. Within the lymphoid lineage, the decision between T cells and B cells is regulated by miR-150 [25, 26].

The early steps of HAEMATOPOIESIS, such as the role of miRNAs in self-renewal of the long-term (LT) HAEMATOPOIETIC STEM CELLS (HSCs) and short-term (ST) HSCs, and the function of miRNAs in mulipotent progenitors, are currently mostly unknown. Due to the low frequency of these cells, it is still very difficult to gain miRNA profiles of such small amounts of cells. Currently, some miRNA expression profiles are available for $CD34^+$ progenitor cells of BONE MARROW and mobilised peripheral blood cells [27] as well



as $CD34^+$ cord blood cells [28]. Furthermore, the miRNA amount in $CD34^+CD133^-$ BONE MARROW cells was quantified (Box 4) [29], showing that among the highest expressed miRNAs in $CD34^+CD133^-$ cells are miR-223, miR-451 and miR-26a with copy numbers of up to 2000 copies per cell.

In summary, up to now some miRNAs and their targets have been identified as important regulators in HAEMATOPOIESIS. In the future, the detailed analysis of miRNAs in cell subtypes and their interaction with other RNA species and proteins will broaden our knowledge about the role of miRNAs in HAEMATOPOIESIS.

Summary

Microarrays are miniaturised devices made for the analysis, for instance, of nucleic acids by HYBRIDISATION. The major benefit of this technology compared to other technologies based on HYBRIDISATION (e.g. Northern blot) is the high degree of parallelisation of TARGET analysis that is realised with this method. Independent of the TARGET (different RNA types, DNA, proteins or other biomolecules), it is feasible to generate a thorough snapshot of a complex situation with a single experiment. DNA microarrays differ with regard to the dispensed probes, the SUBSTRATE (solid phase), the labelling procedure and the process of manufacturing. Apart from *in situ* synthesis of oligonucleotides, the spotting of cDNAs or pre-synthesised oligonucleotides is practised, using CONTACT and NON-CONTACT PRINTING. The amount of sample necessary for HYBRIDISATION depends on the TARGET itself, the labelling method, the type of array and whether the TARGET can be amplified. To give a rough idea, amounts varying from a few pg to 10 µg total RNA are typically required for GENE EXPRESSION analysis. After HYBRIDISATION, the data acquisition consists of two parts: the digitalisation of the signals using scanning devices and the following image analysis using appropriate software packages for quantification of the signals and valid output of primary data. Having generated the primary data, further analysis, like building ratios of the signals when looking at GENE EXPRESSION levels, have to be calculated and normalised. Besides the subsequent interpretation of the expression ratios, further bioinformatics methods can be used to identify relevant TARGET sets for further analysis.

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Recommended websites

Microarray Gene Expression Data Society – MGED Society: Minimum information about a microarray experiment – MIAME: *http://www.mged.org/Workgroups/MIAME/miame.html* (Accessed December 2009)

FIGURE 6. PROMINENT MIRNAS, TRANSCRIPTION FACTORS AND CELL SURFACE MARKERS IN HAEMATOPOIESIS

The miRNAs that regulate the different steps of haematopoiesis are shown in red. The depicted miRNAs were mainly identified with in vitro *assays with human cells. The role of the miRNAs labelled with ‡, e.g. miR-181‡ that drives differentiation towards CLPs, were identified in mouse experiments. The transcription factors are selected according to Orkin and Zon [30].*

LT-HSC, long-term haematopoietic stem cell; ST-HSC, short-term haematopoietic stem cell; MP, multipotent progenitors; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; ErP, erythroid progenitor; MkP, megakaryocyte progenitor; RBC, red blood cell; NK, natural killer.

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Unigene:*http://www.ncbi.nlm.nih.gov/unigene* (Accessed December 2009)

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C

Immunotherapeutics

Vaccines

Gideon F. A. Kersten and Wim Jiskoot

Introduction

Vaccines are the most commonly administered immunotherapeutics. Supported by great improvements in sanitation facilities such as safe drinking water, vaccination was the most effective measure to control a diversity of life-threatening infectious diseases in the 20th century. The most impressive success of vaccination was the global eradication of smallpox in the 1970s. Moreover, the incidence of many other infectious diseases, such as diphtheria, tetanus, pertussis, poliomyelitis, measles, mumps, and rubella, has been drastically reduced thanks to extensive vaccination programmes.

Upon a NATURAL infection with a pathogen, an unprotected person usually falls ill before the immunological defence system is able to respond adequately (see chapter A8). Vaccination aims to stimulate the specific immune response against a pathogen by the administration of attenuated or inactivated organisms, or fractions thereof. If vaccination is successful and the host comes into contact with the pathogen afterwards, the specific immune response will be immediate and sufficiently strong to kill the invading organism before it gets the opportunity to multiply and cause disease. Thus, in a strict sense vaccines are immunoprophylactics rather than therapeutics.

In most cases, repeated doses of the vaccine are given to boost the immune response. Apart from the number of doses, several other factors determine vaccine EFFICACY, as summarised in Table 1. If a high enough proportion of a population is immunised, vaccination not only protects the immunised individuals, but also may help to protect the community as it decreases the chance that non-immunised persons encounter the pathogen. This is referred to as herd immunity [1].

A brief history of vaccination is given below, followed by current vaccine categories for human use. Finally, new developments in vaccinology are outlined.

TABLE 1. FACTORS DETERMINING VACCINE EFFICACY

Pathogen dependent	Host dependent	Vaccine dependent	Vaccination schedule dependent
Port of entry	Species	Nature of antigenic component(s)	Route of administration
Localisation in host	Age	Antigen content	Number of doses
Antigenic variation	Genetic factors	Delivery systems	Immunisation intervals
Mutation frequency	Physical state Immune status	Adjuvants Combination with other vaccine components (in one vial or syringe)	Simultaneous administration of other vaccines (administered separately)

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Historical background

Vaccination has a long history [1, 2]. The most prominent milestones of vaccinology are listed in Table 2. The first attempts to become immune probably date back to as early as the 7th century, when Indian Buddhists drank snake venom and may thus have become immune against this toxin. Written reports bear witness to the practice of variolation, i.e. the administration of scabs or pustule preparations obtained from patients recovered from smallpox, since about 1000 A.D. in various parts of the world, amongst others in China, India, North Africa, and England.

Variolation was widely applied until Edward Jenner introduced cowpox vaccination at the end of the 18th century. His practice was based on the recognition that milkmaids were frequently subjected to mild pox infection acquired from the cows they milked, but were spared from disease during smallpox epidemics. The first demonstration that the principle of immunisation works was Jenner's anecdotal experiment with an 8-year-old boy who remained healthy when challenged with smallpox virus after he had been immunised with cowpox virus. It

Table 2. Milestones in vaccine history[1]

Year	Event[2]
ca. 1000	Intranasal administration of preparations of scabs from smallpox patients in China
16–17th century	Parenteral variolation in India by Hindus
17th century	Oral administration of white cow flea pills for smallpox prevention in China
1796	Immunisation of 8-year old boy with cowpox virus and challenging with smallpox virus (Edward Jenner)
1798	Initiation of general cowpox immunisation with Jenner's variola vaccine
1870s	Discovery of attenuation of fowl cholera bacteria (Louis Pasteur)
1884	Attenuated *Vibrio cholerae*: the first bacterial vaccine used in humans (Robert Koch)
1885	First administration to humans of attenuated rabies vaccine (Louis Pasteur)
1896–1897	Introduction of the first heat-inactivated vaccines against typhoid, cholera and plague
1923	Introduction of the first subunit vaccine: formaldehyde-treated diphtheria toxin
1927	Introduction of BCG, attenuated tuberculosis vaccine
1955	Introduction of inactivated poliovirus vaccine (Salk): the first vaccine developed with tissue-culture technique
1961	Attenuated polio vaccine (Sabin) as the first licensed oral vaccine
1980	Declaration of the eradication of smallpox by the WHO
1986	Licensing of the first rDNA vaccine: recombinant HBsAg
1987	Licensing of the first conjugate vaccine against Hib: PRP-T
2006	Licensing of HPV vaccines, preventing cervical cancer

[1] *Sources: [1,2]*
[2] *BCG, bacille Calmette-Guérin; HBsAg, hepatitis B surface antigen; Hib,* Haemophilus influenzae *type b; HPV: human papilloma virus; PRP-T, polyribosylribitol phosphate-tetanus toxoid conjugate vaccine; WHO, World Health Organisation*

was Jenner who introduced the terms "vaccine" for cowpox preparations (derived from the Latin *vacca* = cow) and "vaccination" for the administration thereof. Later, in honour of Jenner, Louis Pasteur generalised the meaning of vaccination to immunisation with agents other than cowpox. During the 19th century vaccination with live cowpox virus became common practice. In the 20th century, vaccinia virus, which is closely related to cowpox virus [1], became widely used as a live vaccine until smallpox was eradicated.

Pasteur gave a new impetus to vaccinology in the last quarter of the 19th century. He showed that the virulence (i.e. infectivity) of pathogens could be reduced by successive passage in culture. Vaccination with attenuated strains thus obtained could confer protection without causing disease. The efforts of Louis Pasteur and others led to the development of live ATTENUATED VACCINES against cholera, anthrax, and rabies. Along with the introduction of ATTENUATED VACCINES, it became apparent that infection with live material was not essential to induce immunity. The procedure of killing bacteria by heat and subsequent stabilisation with phenol was developed, resulting in the introduction of heat-inactivated whole-cell vaccines against cholera, typhoid and plague at the end of the 19th century.

In the first half of the 20th century the development and introduction of new live [tuberculosis (TB), yellow fever] and inactivated vaccines (pertussis, influenza, rickettsia) followed. Moreover, it was being recognised that some components of a micro-organism were more relevant for protection than others, and the concept of SUBUNIT VACCINES was born. This and the discovery of chemical inactivation of bacterial toxins with formaldehyde led to the introduction of SUBUNIT VACCINES against diphtheria (1923) and tetanus (1927).

In the early 1950s tissue-culture techniques for virus propagation were developed. This resulted in the licensing of Salk's inactivated polio vaccine (IPV) in 1955. In the same period Sabin developed an oral polio vaccine (OPV) consisting of live attenuated viruses, which became available in the USA in 1961. Several other viral vaccines derived from tissue-cultures followed. Furthermore, since the 1970s, several bacterial SUBUNIT VACCINES based on purified proteins or polysaccharides have been introduced. The first vaccine based on rDNA technology, hepatitis B vaccine, was marketed in 1986.

Current vaccine categories

Classification

The currently available vaccines for human use are of either bacterial or viral origin and can be divided into several categories (see Tab. 3). These categories are discussed below. For a more detailed description of individual vaccines currently in practice, the reader is referred to the textbook of Plotkin et al. [1].

Live attenuated vaccines

The first and most successful vaccine was a live vaccine. Cowpox virus was used to eradicate smallpox. It was naturally attenuated because humans are not easily infected by cowpox virus and do not get ill. When human pathogens are used, attenuation through serial passage and selection of less virulent and less toxic variants has been applied to obtain safe vaccine strains. Genetic approaches to attenuation are the use of reassortment techniques and reverse genetics (Box 1). Once a suitable strain has been obtained, master and working seedlots are prepared. The seedlot system provides the basis for the reproducible production of live (and other) vaccines. The dose of live vaccines is determined on the basis of the number of viable organisms.

Live vaccines have a number of advantages over non-living vaccines. Although attenuation generally means reduced infectivity, attenuated strains will replicate to some extent in the recipient. This furnishes a sustained antigen dose, inducing strong immune responses even after a single dose. In general, live vaccines generate higher cell-mediated immune responses than inactivated vaccines. Immunisation with a live vaccine can provide lifelong immunity.

The major drawback of live vaccines is the risk of reversion to pathogenicity. For instance, the occurrence of vaccine-associated paralytic poliomyelitis

Table 3. Classification and examples of current vaccines

Category	Example	Vaccine characteristics
Live attenuated organisms		
viral	Poliovirus (Sabin)	Attenuated viruses, serotypes 1–3; oral vaccine
	Measles virus	Attenuated virus
	Mumps virus	Attenuated virus
	Rubella virus	Attenuated virus
	Yellow fever virus	Attenuated virus
bacterial	Bacille Calmette-Guérin	Attenuated *Mycobacterium bovis*
	Salmonella typhi	Attenuated bacteria, oral vaccine
Inactivated whole organisms		
viral	Poliovirus (Salk)	Formaldehyde-inactivated viruses, serotypes 1–3
	Rabies virus	β-Propiolactone-inactivated virus
	Hepatitis A virus	Formaldehyde-inactivated virus
	Japanese B encephalitis virus	Formaldehyde-inactivated virus
bacterial	*Bordetella pertussis*	Heat-inactivated bacteria
	Vibrio cholerae	Phenol-inactivated bacteria
	Salmonella typhi	Heat-inactivated bacteria
Subunit vaccines		
viral	Influenza virus	Influenza surface antigens
	Hepatitis B virus	Recombinant hepatitis B surface antigen
bacterial	*Corynebacterium diphtheriae*	Formaldehyde-treated toxin
	Clostridium tetani	Formaldehyde-treated toxin
	Bordetella pertussis	Mixture of purified proteins
	Neisseria meningitidis	Purified capsular polysaccharides
	Streptococcus pneumoniae	Purified capsular polysaccharides
	Haemophilus influenzae type b	Polysaccharide-protein conjugates

after the introduction of OPV has been reported [1, 3]. Furthermore, live vaccines sometimes cause mild symptoms resembling the disease caused by the pathogen. Live vaccines should never be given to immunosuppressed persons, because they lack the ability to respond even to infections by attenuated organisms.

Attenuated viral vaccines

Examples of live viral vaccines are polio, measles, mumps, rubella and rotavirus vaccines. Attenuated polio vaccine is administered orally. OPV contains the three existing serotypes, which differ from each other in a number of distinct EPITOPES relevant for

Box 1. Reassortment and reversed genetics

Genetic reassortment is a method to obtain hybrid virus strains by co-infection of cells. The co-infection will lead to progeny virus containing many gene combinations. The virus strain with the desired properties (high immunogenicity, low pathogenicity) is selected for vaccine production. The method is a standard technique for obtaining influenza vaccine strains (also for inactivated vaccines). In that case reassortants are made by injecting an egg with two virus strains: the pathogenic strain and a strain which is harmless for humans but grows well in eggs.
In reverse genetics the genes responsible for pathology or infectivity are changed or deleted. Host cells are transfected with plasmids containing viral genes. In this way progeny virus with the desired properties can be produced. This is the opposite to forward genetics, which is the classical way of obtaining mutants by selection pressure, resulting in phenotypic mutants, in this case attenuation of pathogenicity.

protection. OPV plays an important role in the eradication of poliomyelitis, pursued by the WHO, because it is very effective and relatively cheap. However, the vaccine has a tendency to revert to neurovirulence causing rare cases of vaccine-associated poliomyelitis. Therefore, in some countries OPV is being replaced by IPV (see section 'Inactivated whole organisms').

Attenuated mumps, measles and rubella viruses are often combined in a COMBINATION VACCINE (MMR vaccine). These attenuated RNA viruses vary in size and number of structural proteins. Measles, mumps and rubella vaccines, whether separate or combined, are lyophilised preparations. The three vaccine components have in common that one single s.c. administration is probably sufficient for lifelong protection. Nevertheless, in some countries the first dose given at 12–15 months of age is followed by a second vaccination at the age of 4–6 or 11–12 years. Both humoral and CELL-MEDIATED IMMUNITY are important for protection. Vaccine EFFICACY is estimated to be at least 90% and combining the components does not seem to influence their effectiveness. Side effects are generally mild and usually occur 7–12 days after vaccination. MMR vaccines are not indicated for infants below the age of 1 year, because circulating maternal ANTIBODIES impair vaccine EFFICACY in this age group.

The above-mentioned vaccines are attenuated by classical methods, i.e. repeated passage in cell culture. This is also the case for one of the two live attenuated rotavirus vaccines on the market. Rotarix is based on a single rotavirus strain from human origin attenuated by multiple passages in cell culture. Its competitor, Rotateq, contains five attenuated strains obtained by genetic reassortment (Box 1) of human and bovine strains. The first rotavirus vaccine, based on a human-rhesus reassortant and licensed in 1998, was withdrawn due to a rare but serious side effect: intussusception of the gut. To rule out this possibility, the new generation products were tested in very large clinical trials.

Attenuated bacterial vaccines

The most well-known attenuated bacterial vaccine is TB vaccine, which has been incorporated in many immunisation programmes as of the 1930s. The vaccine is based on *Mycobacterium bovis* bacteria, which primarily infect cattle but can also infect humans. The vaccine consists of lyophilised attenuated *M. bovis*, known as bacille Calmette-Guérin (BCG), and is administered i.d. to infants and older children. The vaccine is not effective in preventing pulmonary TB but it can protect against disseminated TB in young children. Current vaccine strains vary in the extent of attenuation and the dosage varies among vaccine suppliers. The immunisation schedule varies significantly among nations. The nature of the immune response is not known in detail, but cell-mediated immune mechanisms are probably involved in protection, whereas ANTIBODIES do not seem to play a substantial role. There is a great need for effective TB vaccines because strains emerge that are resistant to all antibiotics. The development

of genetically modified live attenuated strains is one line of research [4].

Oral attenuated *Salmonella typhi* vaccines are indicated for high-risk groups, such as children in endemic areas and travellers, to prevent typhoid fever. The only licensed strain is Ty21a, whose attenuation has been stimulated using nitrosoguanidine, a chemical mutagenic agent. Strain Ty21a lacks the ability to synthesise capsular polysaccharides, which are essential for virulence. To protect the bacteria against peptic digestion, the vaccine is formulated as lyophilised bacteria in enteric-coated capsules. Protection is achieved through three to four doses administered every other day. The vaccine provides significant protection by inducing relatively strong intestinal IgA and cell-mediated responses, and a weak systemic ANTIBODY response. Protective ANTIBODIES are directed against flagelli and LPS. The duration of the protection is relatively short (3–5 years).

Inactivated whole organisms

Inactivated bacterial and viral vaccines are obtained from virulent strains by heat treatment or by chemical inactivation, usually with formaldehyde or beta-propiolactone. Since inactivated pathogens are not able to propagate after administration, these vaccines usually are less immunogenic than live vaccines and higher antigen doses are needed. An advantage over the latter is the inability to revert to virulence. On the other hand, deficient inactivation has caused vaccine-related accidents. For instance, immunisation with insufficiently inactivated polio vaccine in 1955 resulted in cases of paralytic disease [1, 2]. Adaptations in the inactivation procedure, like transfer to a new container to guarantee that all liquid is in contact with the formaldehyde and a filtration step to remove possible viral aggregates, guarantee complete inactivation. Examples of this category include inactivated polio vaccine (IPV) and whole-cell pertussis vaccine, which are discussed below.

Inactivated polio vaccine

IPV is currently used in several countries. The vaccine consists of formaldehyde-inactivated poliovirus and includes the three serotypes. The dose is determined on the basis of antigen contents. Advantages of IPV over OPV are a better temperature stability, the absence of vaccine-related disease and the possibility of combination with diphtheria, tetanus and pertussis components in one formulation (DTP-IPV vaccine). In contrast to OPV, IPV does not elicit secretory IgA ANTIBODIES, but its effect relies on the induction of virus-neutralising serum IgG. Because IPV does not replicate *in vivo*, the dose needs to be about ten times higher as compared to OPV, increasing cost and decreasing production capacity. For that reason IPV is currently only used in industrialised or relatively small countries. Nevertheless, to be able to eradicate polio, it is probably necessary to switch from OPV to IPV.

Whole-cell pertussis vaccine

Pertussis vaccine consists of heat-inactivated *B. pertussis* cells. *B. pertussis* bacteria cause whooping cough. The dose is determined on the basis of the opacity of the inactivated cell suspension. The vaccine potency is tested by protection assays in mice. This is a crude and animal unfriendly test involving intracerebral challenge and death as end point. Alternatives, like serological models, are under development. The protective EFFICACY of whole-cell pertussis vaccines is probably based on ANTIBODIES against several pertussis ANTIGENS, such as pertussis toxin, filamentous haemagglutinin and LPS. Whole-cell pertussis vaccines are notorious for their frequent side reactions, mostly fever and irritability. Other side reactions include excessive sleeplessness, persistent inconsolable crying and SHOCK-like phenomena. Although safer acellular pertussis vaccines are on the market (see next section), whole-cell pertussis vaccines are still used in developing countries. The reason for this is mainly economical. Whole-cell vaccines can be produced relatively easily by local manufacturers and are free of intellectual property rights. The adverse effects of whole-cell pertussis vaccines are largely due to the LPS present in the outer membrane of *B. pertussis*. The adverse effects are stronger in older children and adults, so that whole-cell pertussis vaccines are not indicated for these age groups. Although protection is probably

restricted to a period of about 10 years, this is not a significant problem, because pertussis infections are most dangerous in infants. Adolescents and adults in general (but not always) have relatively mild symptoms. The vaccine contains colloidal aluminium salt as adjuvant and is usually combined with diphtheria and tetanus vaccine components (DTP vaccine). The vaccine is given in four to five i.m. doses.

Subunit vaccines

SUBUNIT VACCINES contain one or more selected ANTIGENS (subunits) significant for protection against the pathogen they are derived from. SUBUNIT VACCINES have better defined physicochemical characteristics and show less side effects than vaccines consisting of attenuated or inactivated organisms. ANTIGENS used for current SUBUNIT VACCINES include viral and bacterial proteins as well as bacterial capsular polysaccharides.

Proteins

Protection against *Corynebacterium diphtheriae* or *Clostridium tetani* is based on the presence of ANTIBODIES directed against the respective toxins. These toxins are water-soluble proteins and form the basis of diphtheria and tetanus vaccines. To eliminate the toxicity of diphtheria and tetanus toxin, they are incubated with formaldehyde. This process is called toxoidation and the resulting products are referred to as toxoids. Formaldehyde forms covalent bonds with the toxin, which is initiated by a reversible reaction of formaldehyde with primary amino groups, followed by an irreversible reaction with other amino acid residues [2]:

$$R\text{–}NH_2 + CH_2O \leftrightarrow R\text{–}NH\text{–}CH_2OH \leftrightarrow R\text{–}N = CH_2 + H_2O \quad (1)$$

$$R\text{–}N = CH_2 + R'\text{–}H \rightarrow R\text{–}NH\text{–}CH_2\text{–}R' \quad (2)$$

where R is the toxin and R' can be a reactive amino acid residue (e.g. lysine, arginine, tryptophan, tyrosine, histidine) in the toxin molecule (or possibly a neighbouring toxin molecule) or a free amino acid present in the matrix. Thus, stable cross-links are formed, yielding a HETEROGENEOUS product with respect to number and sites of formaldehyde adducts and molecular weight. The degree of toxoidation is highly dependent on the reaction conditions, including formaldehyde concentration, pH, temperature and the presence of other components. The toxoidation process must be a compromise between sufficient detoxification and preservation of relevant EPITOPES. To enhance the relatively poor immunogenicity of toxoids, they are adsorbed to aluminium salt suspensions.

Acellular pertussis vaccines were introduced in Japan in the early 1980s as alternatives to the whole-cell vaccines for the immunisation of older children. Although pertussis toxoid alone may be sufficient for protection, most acellular pertussis vaccines contain at least two proteins important for the virulence of *B. pertussis*, including (inactivated) pertussis toxin, filamentous haemagglutinin, fimbriae and pertactin. Field trials indicate that the EFFICACY of these vaccines is comparable to that of whole-cell vaccines, whereas the acellular vaccines induce virtually no adverse effects [5]. This makes them suitable for immunisation of older children and adults. Acellular pertussis vaccines have now been introduced in many national immunisation programmes, mostly combined with diphtheria and tetanus components (DTaP vaccine).

Virus-like particles, virosomes and vesicles

To induce an ANTIBODY response, an antigen has to be accessible to B cells. Therefore, ANTIGENS eliciting protective ANTIBODY responses are often membrane proteins. In the presence of lipids these ANTIGENS can spontaneously form virus-like particles (VLPs). VLPs are usually very immunogenic because of the multimeric presentation form.

Hepatitis B vaccine was the first marketed RECOMBINANT vaccine, and has replaced hepatitis B vaccines obtained from plasma of infected humans. RECOMBINANT hepatitis B vaccines are composed of hepatitis B surface antigen (HBsAg) derived from yeast or mammalian cells. Purified HBsAg and lipids self-assemble to 22-nm particles identical to those excreted by cells infected with the native virus. The ease of production has made the vaccine available

worldwide and in many countries the vaccine is given to all infants.

Virosomes are lipid vesicles extracted from membrane viruses by detergent treatment. They contain membrane-associated viral ANTIGENS and often induce potent immune responses. An intranasal influenza virosomal vaccine was used during the 2000–2001 influenza season but had to be withdrawn because it caused Bell's palsy (temporary paralysis of one side of the face) in rare cases. This was presumably caused by the adjuvant it contained and not the virosomal formulation as such.

Currently two human papillomavirus (HPV) vaccines are on the market: Gardasil from Merck and Cervarix from GSK. Both are based on RECOMBINANT VLPs. Gardasil is a quadrivalent vaccine and Cervarix contains VLPs from two virus types. The vaccines protect against cervical cancer. It has been shown in phase III clinical trials that the bivalent Cervarix vaccine not only protects against the two virus types present in the vaccine but also cross protects against three other important cancer-inducing strains [6].

Capsular polysaccharides

Many bacteria have a CAPSULE consisting of high-molecular-weight polysaccharides, which act as virulence factors. CAPSULE-forming species include both GRAM-POSITIVE (e.g. pneumococci) and Gram-negative BACTERIA (e.g. meningococci). The polysaccharides of the different species are composed of linear repeat oligosaccharide units that vary in sugar composition and chain length. The host defence against encapsulated bacteria relies on anti-polysaccharide ANTIBODIES interacting with complement to opsonise the organisms and prepare them for PHAGOCYTOSIS and CLEARANCE. Licensed capsular polysaccharide vaccines include meningococcal (serogroups A, C, W-135, Y), pneumococcal (up to 23 serotypes) and *Haemophilus influenzae* type b (Hib) vaccines.

The main disadvantage of capsular polysaccharide vaccines is their T cell independency, which implies that they do not elicit immunological memory. Moreover, infants up to 2 years of age show very weak, non-protective immune responses, whereas they belong to the highest risk groups for infections with the encapsulated bacteria mentioned above.

Polysaccharide-protein conjugate vaccines

The poor immunogenicity of plain polysaccharides can be overcome by covalent coupling to CARRIER proteins containing T cell EPITOPES. These helper EPITOPES make them T cell dependent and enables the induction of strong immune responses and immunological memory in all age groups, including infants. Conjugate vaccines licensed so far are Hib, meningococcal group C and pneumococcal vaccines. The Hib polysaccharide consists of repeat units of ribosyl(1-1)ribitol phosphate. An effective *H. influenzae* vaccine is relatively easy to produce, because – in contrast to the diversity of pathogenic meningococcal and pneumococcal strains – Hib is responsible for about 95% of infections with *Haemophilus* species, so only one polysaccharide type has to be included in the vaccine. Table 4 shows that the four licensed Hib conjugate vaccines vary in composition, owing to differences in polysaccharide length, CARRIER protein, coupling procedure and polysaccharide-to-protein ratio. As a result, these vaccines differ with respect to immunogenicity and EFFICACY [7]. The vaccines are incorporated in many childhood immunisation programmes and are normally administered i.m. in a multi-dose schedule with DTP, either separately or as a combined DTP-Hib vaccine. In some countries Hib is combined in a pentavalent (DTP-IPV-Hib) or hexavalent (DTP-IPV-Hib-HepB) COMBINATION VACCINE. Another example of a licensed conjugate vaccine is a mixture of polysaccharides from seven types of pneumococci conjugated to diphtheria toxin. This vaccine, apart from being a medical success, was also commercially attractive, causing renewed interest of pharmaceutical companies in vaccine development. The pneumococcal vaccine is now also available as a 13-valent vaccine. Finally, several manufacturers produce meningococcus group C conjugate vaccine.

Pharmacological effects of vaccination

The EFFICACY of a vaccine is difficult to estimate, because the relationship between immune response and degree of protection is not straightforward.

Table 4. Characteristics of licensed *H. influenzae* type b (Hib) conjugate vaccines[1]

	Vaccine[2]			
Property	**PRP-D**	**HbOC**	**PRP-OMP**	**PRP-T**
Polysaccharide size	Medium	Small	Medium	Large
Polysaccharide content (μg)	25	10	15	10
Carrier protein	Diphtheria toxoid	Diphtheria toxin mutant	Meningococcal group B outer membrane proteins	Tetanus toxoid
Protein content (μg)	18	20	250	20
Linkage	Via spacer	Direct	Via spacer	Via spacer
Formulation	Aqueous solution	Aqueous solution	Lyophilised, reconstituted with alum salt suspension	Lyophilised, reconstituted with aqueous buffer

[1]*Source: [1]*
[2]*PRP, polyribosylribitol phosphate; PRP-D, PRP-diphtheria toxoid conjugate vaccine; HbOC,* Haemophilus *type b oligosaccharide conjugate vaccine; PRP-OMP, PRP-outer membrane protein conjugate vaccine; PRP-T, PRP-tetanus toxoid conjugate vaccine*

Seroconversion, i.e. the increase in the level of specific circulating ANTIBODIES, is commonly determined as a measure for the immunogenicity (see chapter B1). Moreover, the protective quality of these ANTIBODIES can be measured with assays for bactericidal activity, i.e. their ability to kill bacteria in the presence of complement (e.g. meningococcal vaccines), virus-neutralising activity (e.g. polio vaccines) or toxin-neutralising activity (e.g. diphtheria vaccines). However, it is hard to correlate the level and persistence of circulating ANTIBODIES to protective EFFICACY. For some vaccines, like tetanus and diphtheria toxoid, and Hib and meningococcal type C vaccine, serological correlates of protection have been established [8]. However, the extent of CELL-MEDIATED IMMUNITY may in some instances be a better measure for protection, e.g. against TB, some viral diseases and therapeutic tumour vaccines. Advances in the measurement of cell-mediated immune responses enable vaccine developers to measure cellular immunity in small blood samples. T cells can be detected with sensitive methods like FLOW CYTOMETRY (see chapter B3) after cytokine staining or staining with fluorescent HLA-tetramers. Antigen-specific LYMPHOCYTES can be detected by the ELISPOT technique, in which cytokine-producing cells are detected in plates coated with anti-cytokine ANTIBODIES. The effectiveness of vaccination is most clearly demonstrated by the reduction of disease after introduction of a vaccine in national immunisation programmes. Recent examples are the drastic reduction in incidence of Hib infections observed in those areas where routine vaccination in infants was introduced and a similar effect after the introduction of meningococcus group C vaccination in the UK. There is much indirect evidence of vaccine EFFICACY. For instance, in The Netherlands, where the use of IPV has effectively protected most of the population, two significant outbreaks of poliomyelitis in 1978 and 1992 were restricted to communities that refused vaccination on religious grounds. The number of measles cases dropped dramatically after introduction of MMR vaccine. Due to a reported link between MMR vaccination and autism, later proved to be wrong [9], vaccination coverage dropped in the UK to a low of 80% in 2003–2004. As a result the number of cases of this highly contagious disease increased strongly.

Since the TARGET groups of vaccines in many cases include healthy infants and young children, vaccine safety is of particular importance. The occurrence of side effects may be due to the antigenic components (e.g. LPS in whole-cell pertussis vaccine), impurities derived from the production process, (e.g. chick protein from the cell SUBSTRATE used for measles vaccine production), or to additives used in a vaccine formulation (e.g. neomycin or gelatin in MMR vaccines, aluminium salts in SUBUNIT VACCINES). Before a new vaccine candidate is licensed, its safety is investigated in animals and in humans in phase I, II and III clinical trials. Phase I trials include a small number of healthy adults and serve to collect preliminary safety data. In phase II studies, safety and immunogenicity are determined in a larger number of volunteers, usually in the TARGET population. Vaccine dosage is also assessed. Phase III trials are meant to evaluate safety, including rare adverse effects, and EFFICACY in large TARGET populations.

New developments

Introduction

Notwithstanding the success of vaccination, several infectious diseases remain against which an effective vaccine is not yet available. New vaccines against bacterial (e.g. group B meningococci), viral (e.g. HUMAN IMMUNODEFICIENCY VIRUS) and parasitic (e.g. malaria) infections are under development. Ideally, these vaccines should provide lifelong protection in any individual of any age, be absolutely safe, easy to produce in unlimited quantities, stable under varying conditions, easy to administer and cheap. As yet the design of a vaccine with all these ideal characteristics combined remains an important challenge for developers of new and better vaccines.

Apart from new prophylactic vaccines, current research is also focused on the development of therapeutic vaccines, especially for the treatment of chronic diseases such as AIDS and cancer. The rationale of administering vaccines to patients already suffering from disease is to specifically boost the IMMUNE SYSTEM weakened by the disease.

The number of vaccines routinely applied is expected to increase, which demands efforts to reduce the number of injections. An obvious way to achieve this is combining separate vaccine components into one vial or syringe. Examples of such COMBINATION VACCINES have been given before. Simply mixing vaccine components, however, may not only pose pharmaceutical problems (e.g. incompatibility of vaccine components and/or excipients), but also bears the risk of immunological interference. For instance, hepatitis B vaccine was reported to be less immunogenic when mixed with DTaP-IPV-HiB vaccine [10].

Modern technologies

Whereas traditional vaccine development has largely been dependent on empirical methods, a better insight into immune mechanisms and immunogenic structures of infectious organisms has led to a better understanding of what would be the optimal vaccine composition as related to the desired immunological effect. Recent advances in genomics, PROTEOMICS and bioinformatics are now being applied to identify putative antigen (Box 2) [11]. Moreover, the advent of (bio)technological advances has enabled scientists to translate the improved immunological knowledge into the rational design of new vaccines against a variety of life-threatening and chronic diseases. Several classical and modern approaches to the development of a variety of new vaccines are currently being explored, some of which are schematically shown in Figure 1. Most of these approaches offer the following common advantages over classical vaccines: (i) relevant EPITOPES of pathogenic organisms or cancer cells are obtained by safer means and (ii) in greater quantities, (iii) the products are better defined, and (iv) EPITOPES of a single or multiple pathogenic agents can be combined easily in one vaccine. Approaches not yet addressed before are briefly discussed below. For more detailed information about modern vaccine technology the reader is referred to specialised textbooks [1,2].

Box 2. Identification of antigens

One of the difficult tasks in the development of subunit vaccines is the selection of the relevant antigen(s) (Ag). Apart from being able to induce protecting immmune responses, they need to be conserved in different strains of a pathogen to provide broad protection. Also the Ag must be stable under immune pressure to prevent vaccination-induced selection.

A genomics approach to screen for leads was coined reversed vaccinology [8]. Once the genome of a pathogen, for instance a bacterium, is sequenced, possible vaccine candidates can be selected by *in silico* searching for sequences coding for transmembrane sequences or sequences coding signal peptides for extracellular transport. This will reveal proteins that would be exposed to the immune system, containing B cell epitopes. These genes are cloned in plasmids and recombinant proteins are produced. Immunisation studies will reveal whether the proteins are immunogenic and induce functional antibodies. The number of candidates is further reduced by looking for sequence variability within clinical isolates of the pathogen, selecting for conserved Ag.

T cell epitopes presented by MHC molecules can be identified by mass spectrometric techniques [12]. APCs are incubated *in vitro* with Ag or a pathogen. The Ag is processed and antigenic peptides are presented by MHC molecules at the cell surface. MHC molecules are isolated and the bound peptides are eluted. Finally, the amino acid sequence of the peptides is determined by tandem mass spectrometry. To distinguish relevant peptides from self peptides, Ag can be used that is labelled with heavy isotopes, for instance ^{15}N. When a mixture of Ag containing the natural and the heavy isotope is used, the relevant (i.e. non-self) peptide masses will appear as doublets in the mass spectrum. These doublets are easily identified, speeding up identification.

Recombinant live vaccines

Non-pathogenic or attenuated organisms can be used as carriers for heterologous protein ANTIGENS. Such live carriers are called vectors (Fig. 1C). They are obtained by cloning the desired gene and introducing it into an appropriate CARRIER organism. Both viral (e.g. vaccinia virus, adenoviruses) and bacterial vectors (e.g. *Salmonella* species, BCG) are being explored as CARRIER to express a variety of ANTIGENS. The properties of RECOMBINANT live vaccines are comparable to those of classical ATTENUATED VACCINES.

Fusion proteins

Fusion proteins are non-toxic proteins containing inserted EPITOPES, larger protein fragments or even entire proteins derived from pathogenic species (Fig. 1E). They are obtained by the insertion of DNA sequences encoding EPITOPES in the gene of the CARRIER protein, such as HBsAg or a fusion partner with the capability to TARGET the fused antigen to antigen-presenting cells (APCs) [13]. The RECOMBINANT gene is expressed in a suitable organism and the fusion protein is then purified. A drawback of this genetic fusion technology is potential misfolding of the epitope when incorporated in the CARRIER protein, which would lead to irrelevant immune responses.

Synthetic peptide vaccines

Chemically synthesised peptides belong to the best-defined vaccine components presently under investigation. The SYNTHETIC peptide technology allows for the design of vaccines consisting of selected EPITOPES free from irrelevant or unwanted structures. Large amounts of linear peptides resembling T cell or B cell EPITOPES (Fig. 1G) can be prepared by automated methods. The immunogenicity of SYNTHETIC peptide antigen is weak, but can be enhanced by conjugation to CARRIER proteins (analogous to polysaccharide-protein conjugates) or to lipids, or by the construction of multiple different B and T cell EPITOPES. These options offer the possibility to render SYNTHETIC B cell EPITOPES T cell dependent. Furthermore, the conformational freedom of small linear peptides

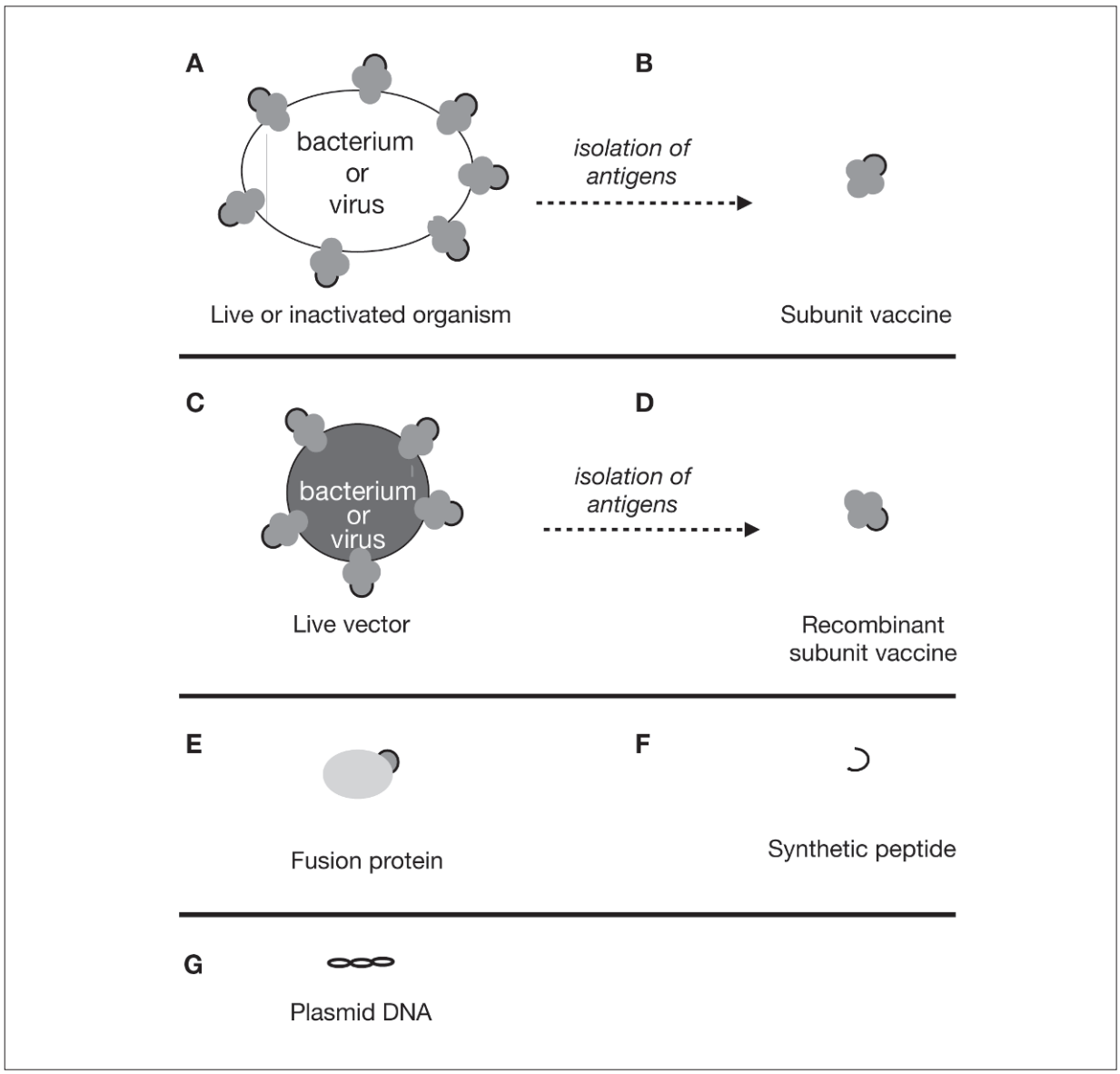

Figure 1. Schematic representation of (A, B) classical vaccine components and (C–G) new generation vaccine components

(A) Whole bacterium or virus, live attenuated or inactivated, with protein antigens (grey objects) containing protective epitope (black semi-circle); (B) subunit vaccine: antigen isolated from pathogenic organism; (C) live vector: antigenic proteins derived from pathogenic organism expressed by live, non-pathogenic bacterium or virus; (D) recombinant subunit vaccine: antigenic protein isolated from heterologous expression system; (E) fusion protein: non-toxic protein containing epitope of protein from pathogen isolated from non-pathogenic organism; (F) synthetic peptide with amino acid sequence mimicking epitope of antigen from pathogen; (G) nucleic acid vaccine: plasmid DNA containing the gene encoding antigenic protein or epitope from pathogen.

can be restricted by cyclisation [14], which aims to force them into a conformation reflecting the native structure, which is especially important for peptide analogues of B cell EPITOPES [15].

Nucleic acid vaccines

A development of potential clinical use is genetic immunisation, i.e. direct administration of DNA encoding one or more ANTIGENS of interest (Fig. 1H). Upon i.m. immunisation with non-replicating plasmid DNA, the protein encoded is produced and expressed by the host cell. RNA may also be used, but is less suitable because it is rapidly degraded *in vivo* and more expensive to produce. Nucleic acid vaccines are capable of eliciting both humoral and cellular immunity. Induction of cellular immunity is particularly important for immunisation against retorviruses such as HIV and HCV. A sustained production of antigen(s) after a single immunisation is expected to provide long-term protection [16].

Route of administration

The EFFICACY of vaccination programmes would be enhanced greatly through the availability of needle-free immunisation methods. Injections with needle and syringe are not appreciated by most people and about 10% of the population avoids injections. Furthermore, needlestick injuries and re-use of needles still cause many infections of hepatitis B and HIV. Several alternative routes of administration have therefore been explored, including mucosal (oral, intranasal, pulmonary) or dermal immunisation.
Mucosal immunisation routes are attractive alternatives to parenteral routes, not only because of the ease of administration, but also because both systemic and mucosal (secretory IgA) responses are induced. The latter is advantageous, because mucosal surfaces are the common port of entrance of many organisms and a strong local immune response may hamper entry into the host by preventing ADHERENCE to and colonisation in mucosal surfaces. However, although mucosal immunisation is among the oldest means of vaccination (see Tab. 2), the number of vaccines suitable for mucosal immunisation is limited to a few oral vaccines and a live attenuated influenza vaccine called Flumist. Poliovirus can be given orally because the virus is relatively resistant to low pH, whereas oral typhoid vaccine is protected from gastric breakdown by formulation in enteric-coated capsules. Other mucosal administration routes, such as nasal or pulmonary delivery, have the advantage that the harsh conditions in the gastrointestinal tract are circumvented. Approaches to augment the immunogenicity of future mucosal vaccines include the use of vectors (e.g. *Salmonella*) or VACCINE DELIVERY SYSTEMS and co-administration of adjuvants [17].

TOPICAL administration of ANTIGENS is an appealing delivery route because the skin is a very immune active organ containing a large number of specialised APC, the Langerhans cells and dermal DENDRITIC CELLS [18]. The skin was for a long time considered almost impermeable for large ANTIGENS and the only way to deliver to the skin was by shallow injection. Recently a syringe with a 1.5-mm needle for intradermal delivery of 0.1 mL vaccine became available. The small needle length allows injection perpendicular to the skin, making true dermal delivery easier and more reliable. Many other techniques, avoiding classical needles and syringes are under development. Chemical penetration enhancers (e.g. surfactants and liposomes) as well as physical approaches (e.g. iontophoresis) to enhance the permeability of the skin for macromolecules have shown some success.

Glenn and co-workers [19] have shown that TOPICAL application of patches containing heat-labile enterotoxin generates potent immune responses in man. The stratum corneum, the outer layer of dead cells can be made permeable for large molecules with microstructures that, upon wiping the skin, damage it just enough to enable ANTIGENS to diffuse into the skin when applied as a patch afterwards. A vaccine against traveller's diarrhoea based on heat-labile toxin of *E. coli* is in phase III clinical trials.

Other methods to bypass the stratum corneum are the use of microneedles. Arrays of needles of 0.5 mm or less can be used to puncture the skin without causing pain. Microneedles are either massive and are being used to 'pre-puncture' or they can be coated with vaccine. Hollow microneedle arrays capable of injecting a small volume are under development.

Finally, jet injectors are a way of delivering vaccine into the skin. These devices do not contain needles and the vaccine is delivered by forcing it under high pressure through a nozzle. The fluid or powder jet will penetrate the skin and, depending on the jet speed and nozzle design, will be deposited intradermally, subcutaneously or intramuscularly.

Vaccine delivery systems and adjuvants

In general it can be stated that the smaller the size of a vaccine component, the weaker its immunogenicity. Therefore, a lot of effort has been and is being put into enhancing the immune response to SUBUNIT VACCINE components by suitable presentation forms, including sophisticated VACCINE DELIVERY SYSTEMS and adjuvants (see Box 3). CARRIER proteins and live vectors are delivery systems that have been discussed already. Other delivery systems include particulate carriers, such as biodegradable microcarriers, nanoparticles, liposomes, immune-stimulating complexes (ISCOMs) and virosomes. The traditional aluminium phosphate and aluminium hydroxide colloid salts, only stimulate humoral immune responses. Also, they direct the immune response mainly to type 2 (Th2) responses. Often a more balanced Th1/Th2 response is needed for protection. Many novel candidate adjuvants, such as saponins, lipid A derivatives and bacterial DNA sequences containing CpG-oligodinucleotides, also augment cellular immune responses and mediate their effect through non-specific induction of several CYTOKINES, resulting in balanced Th1/Th2 responses. The B subunits of cholera toxin and of *E. coli* heat-labile enterotoxin are examples of powerful mucosal adjuvants. CYTOKINES such as IL-2 and -12 and IFN have become of interest as more specific adjuvants, especially in the search for potent vaccines against AIDS and cancer. Many adjuvants contain PATHOGEN-ASSOCIATED MOLECULAR PATTERNS (PAMP), molecular structures unique to micro-organisms. These PAMPs are ligands for TOLL-LIKE RECEPTORS (TLR) present on or in APCs (see chapter A5). Binding of a PAMP to a TLR activates pathways that result in onset of the innate immune response (see chapter A6).

Until a few years ago, only aluminium phosphate and aluminium hydroxide were used in human vaccines. This has now changed, mainly based on a better understanding of the mechanism of action and an improved balance between adjuvanticity and adverse effects. Even for aluminium salts the mechanism of action has been unravelled in much more detail [20]: apart from a depot effect, alumin-

BOX 3. PRESENTATION FORMS

Adjuvants comprise a large number of substances of variable chemistry and origin. Examples are colloidal salts, lipid matrices, surface-active compounds and emulsions of mineral, bacterial, vegetable or synthetic nature. Adjuvants have in common that they are not immunogenic *per se*, but enhance the immunogenicity of co-administered Ag.

Vaccine delivery systems are colloidal carriers with a size that can vary from ~50 nm to the micrometer range, allowing multimeric Ag presentation at their surface. Thereby they mimic the natural presentation of Ag on viral or bacterial surfaces. In general, multimeric presentation of Ag strongly improves their immunogenicity. Colloidal carriers can function as a depot at the administration site, resulting in sustained delivery and a reduction of the number of doses required. Moreover, they can enhance humoral and/or cellular immune reactions, because colloidal particles are taken up more efficiently by APCs (in particular dendritic cells) as compared to free Ag. Uptake of colloidal particles by dendritic cells can be further promoted by coupling targeting moieties, specifically recognising dendritic cell receptors, to their surface. As M cells present in mucosal membranes are specialised in the uptake of particulate material and subsequent presentation to immune cells, colloidal particles are also suitable Ag carriers when mucosal immunisation is pursued. In addition, they may protect the Ag from proteolytic attack (e.g. in oral vaccine formulations). Besides Ag, adjuvants are sometimes incorporated into these carrier systems, and many carrier systems have intrinsic adjuvant activity.

ium salts activate danger signals by the induction of uric acid. The 2009–2010 pandemic influenza vaccines, Focetria and Pandemrix, contain squalene-based emulsions as adjuvant. The new HPV vaccine Gardasil contains, apart from aluminium salt, monophosphoryl lipid A, a non-toxic analogue of bacterial lipid A, targeting TLR-4, thereby activating APCs.

Summary

Vaccines have been very successful in the prevention of infectious diseases. Traditional vaccines consist of whole (live or inactivated) bacteria or viruses, or components thereof, and are among the oldest biotechnological pharmaceuticals. Several modern approaches, most of which are based on rDNA technologies, are emerging with the aim of generating more effective and safer vaccines. As a result of the tendency to design smaller, better-defined antigenic components, proper antigen presentation forms and the use of adjuvants are becoming increasingly important. New adjuvants are now routinely used in some vaccines and more are under development. Moreover, to limit an increasing number of injections, needle-free immunisation routes are being explored. It is expected that vaccines to be marketed in the near future will be based on some of the modern vaccine technologies discussed in this chapter and – like the conventional vaccines – will have a significant contribution to the improvement of public health.

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Plasma-derived immunoglobulins

Adrian W. Zuercher, Lorenz Amsler, Hanspeter Amstutz, Irmgard Andresen, Reinhard Bolli, Wolfram Hummel, Fabian Käsermann, Christoph Kempf, Peter Lerch, Marius Lötscher, Alexander Schaub, Martin Spycher and Sylvia M. Miescher

Introduction

When the human body is invaded by foreign organisms it defends itself either through the propagation of a cellular response (CELL-MEDIATED IMMUNITY) or by the production of soluble proteins. This production of soluble proteins is known as humoral immunity, so-called because it refers to substances found within the humours, or body fluids. While there are a number of components that have been identified as important in the humoral immune response, including complement proteins [1], the key molecules necessary for mediating specific humoral immunity are IMMUNOGLOBULINS (Ig), also known as ANTIBODIES (Ab) (see also chapter A3).

Ig molecules are complex proteins made up of two identical heavy chains and two identical light chains, linked by disulphide bonds and arranged in a 'Y'-shape (Fig. 1A) [2]. They are expressed on the surface of B cells or secreted, recognising and binding to ANTIGENS (Ag), i.e. 'foreign' or 'dangerous' structures, such as bacterial or viral surface proteins and surface polysaccharides, as well as secreted bacterial toxins. Variable domains in the N-terminal regions of the heavy and light chains recognise and bind Ag, while the constant regions of the heavy chain C-terminal domains mediate effector functions and define the class or isotype of Ig (Box 1) (see also chapter A3). An Ag is recognised by shape. While this may be a continuous peptide or surface polysaccharide, more often it is the three-dimensional shape made when the Ag is folded into its native structure. The shape recognised by the Ig is called an epitope. Each B cell can only produce one specific Ab, which defines its IDIOTYPE and the total number of IDIOTYPES a body can produce is known as the immune REPERTOIRE [3]. The process by which ANTIBODY diversity is generated has been described in detail in chapter A3.

The therapeutic use of Ig has been known for many years. Hyperimmune sera collected from immunised animals, or now more often from immunised or naturally exposed donors, have long been used as a PASSIVE IMMUNOTHERAPY treatment to provide specific Ab to an individual immediately, e.g. after snake envenomation, *Clostridium tetani* infection or rabies infection (Tab. 1) [4, 5]. Similarly, anti-Rhesus D (RhD) Ig has been used successfully in the prevention of haemolytic disease in neonates due to Rhesus incompatibility between mother and child, or after mistransfusion of blood components containing RhD-positive ERYTHROCYTES to RhD-negative recipients. More details on hyperimmune Ig are provided later in this chapter (section "Immunoglobulin preparations for medical use").

For over half a century, Ig has also been used therapeutically in patients with immune deficiencies [6]. Initially administered intramuscularly (i.m.), benefits in terms of EFFICACY and safety saw a switch to intravenous (i.v.) administration of Ig, referred to

Box 1

There are five different classes of Ig: IgG, IgM, IgA, IgD and IgE as well as four subclasses of IgG and two subclasses of IgA; each class has a different function (see Fig. 2).

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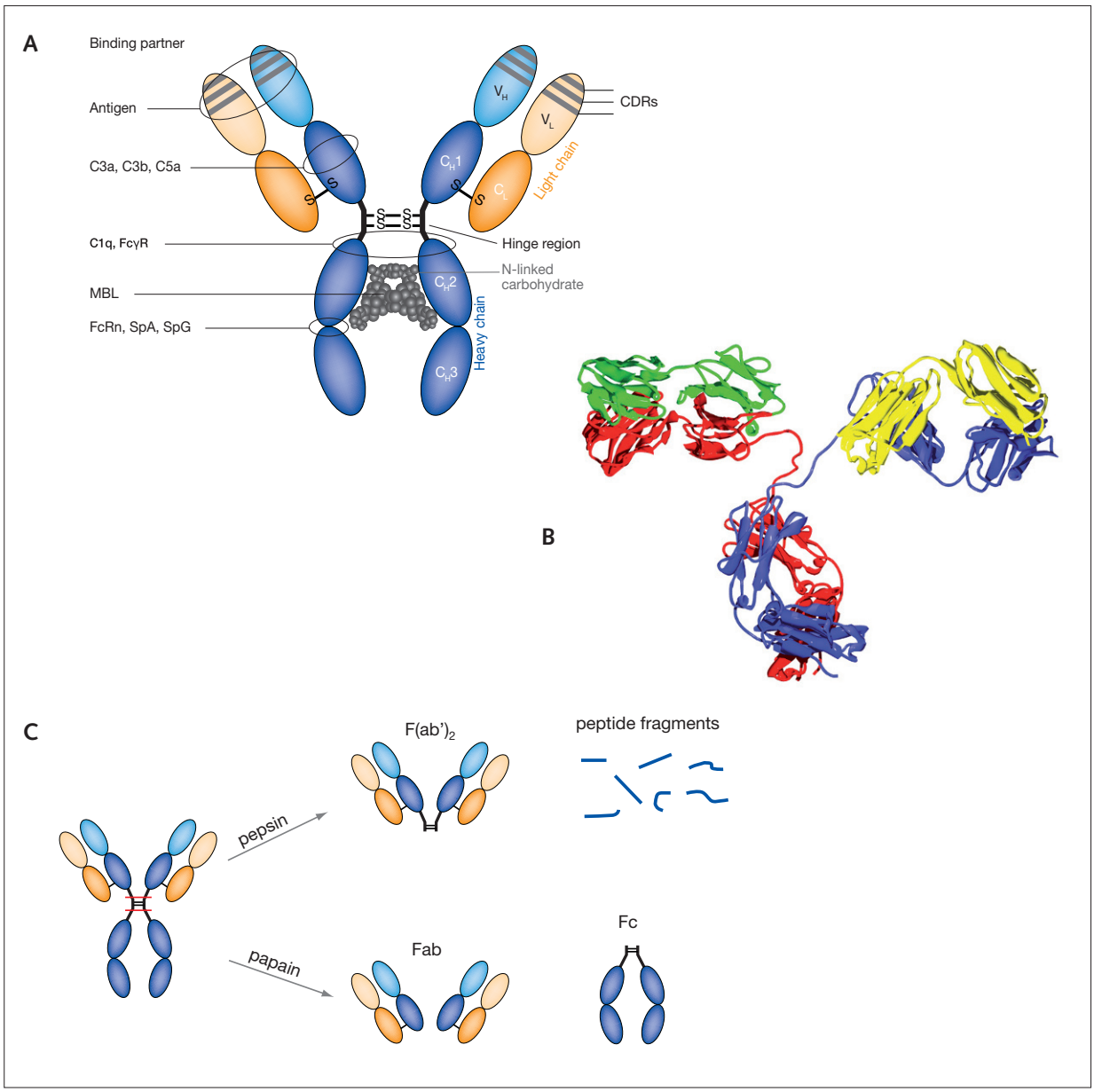

Figure 1. Structure of IgG

(A) Detailed structure of an IgG1 molecule. CDR, Complementarity Determining Region; C_H, constant, heavy; C_L, constant, light; V_H, variable, heavy; V_L, variable, light; S-S, disulphide bonds; C3a, C3b, C5a, C1q, complement proteins; FcγR, Fcγ receptor; MBL, mannan binding lectin; FcRn, neonatal Fc receptor, SpA, Staphylococcus protein A; SpG, Streptococcus protein G. (B) Ribbon structure of an IgG2 antibody. This image has been released worldwide into the public domain by its author, Tim Vickers, at the Wikipedia project. Carbohydrates are not shown in this figure. (C) IgG fragments.

Table 1. High titre Ig preparations against selected Ag

	Origin	Administration	Manufacturer (selection)
Anti-virus			
Cytomegalo	Human	i.m., i.v.	Biotest, CSL, CSL Behring
Hepatitis A	Human	i.m.	CSL Behring
Hepatitis B	Human	i.m., i.v.	Biotest, Cangene, CSL, CSL Behring, Grifols, Kedrion
Rabies	Human	i.m.	CSL Behring
Respiratory syncytial	Human	i.v.	MedImmune
Vaccinia	Human	i.v.	Cangene
Varicella zoster	Human	i.m., i.v.	Biotest, Cangene, CSL, CSL Behring
Anti-bacterial toxins			
Botulism	Human	i.v.	California DHS
Diphtheria	Horse	i.m., i.v.	Haffkine
Tetanus	Human	i.m., i.v.	CSL, CSL Behring, Grifols, Kedrion
Anti-venoms			
Scorpion	Horse	i.v.	Haffkine, SAIMR
Snake	Horse, sheep	i.v.	CSL, Haffkine, SAIMR, Savage Laboratories
Spider	Horse	i.m./i.v.	CSL, SAIMR
Jellyfish	Sheep	i.v.	CSL
Stonefish	Horse	i.m. (i.v.)	CSL
Other Ag			
D (Rho)	Human	i.m., i.v.	Baxter, Cangene, CSL Behring, Grifols, Kedrion
Digitalis	Sheep	i.v.	Roche, Savage Laboratories
Human thymocytes	Rabbit	i.v.	Genzyme

as IVIG. More recently, other routes of administration of Ig have been explored, in particular subcutaneous (s.c.) administration. These are discussed in more detail later in the chapter (section "Pharmacokinetics of IMMUNOGLOBULINS").

IVIG is prepared using IgG isolated from pooled plasma samples usually obtained from 15 000 to 60 000 normal healthy donors [7]. Patients with primary immune deficiency (PID) are unable to produce sufficient amounts of Ig and are therefore at increased risk of bacterial, fungal or viral infections. Thus, IVIG as a replacement therapy is nowadays the standard of care in the treatment of a number of immunodeficiencies such as severe combined immunodeficiency (SCID), X-linked agammaglobulinaemia and Wiskott-Aldrich syndrome.

IVIG is also indicated for the treatment of some AUTOIMMUNE DISEASES, including immune thrombocytopenia (ITP), Kawasaki disease and Guillain-Barré syndrome (GBS) (see below for more details) [8]. The MECHANISMS OF ACTION of IVIG in the treatment of AUTOIMMUNE DISEASES have been the subject of many investigations but are still not clear. Interference with COMPLEMENT COMPONENTS and the cytokine network,

Fc RECEPTOR blockade, presence of anti-idiotypic Ab and modulation of B and T cell functions are all thought to play a role in IVIG EFFICACY and these are discussed further in a later section of this chapter (section "Mechanisms of action").

Antibody structure and effector functions

As mentioned above, all Ig molecules have a common symmetrical core structure made up of two covalently linked heavy chains as well as two light chains, each linked to a heavy chain. Within each chain there are two to five groups of approximately 110 amino acids, which are independently folded and make up the Ig domains, termed V_H, C_H1, C_H2, C_H3, C_H4, V_L and C_L (Fig. 1A, B). These Ig domains contain conserved regions and intrachain disulphide bonds. The Fab (fragment, antigen binding; Fig. 1C) portion of the Ig (the top of the 'Y' shape) is the region responsible for Ag recognition. The Ag is recognised by three hypervariable regions within the N-terminal, or the top, of the heavy and light chains. These hypervariable regions consist of approximately 10 amino acids and are collectively known as the complementarity-DETERMINING REGION (CDR; Fig. 1A). It is the large variability within the CDR that accounts for the wide range of Ag shapes recognised by Ig molecules. Once the Ag is bound to the CDR, the C-terminal regions at the base of the heavy chain, which have a uniform or constant structure, can then mediate the effector functions of the Ig molecule (see also Box 2). Known as the crystallisable fragment (Fc), the effector portion of the Ig can mediate a number of different reactions (Tab. 2 on pp. 276/277; Fig. 1C). Digestion by papain or pepsin yields either two Fab fragments and an Fc fragment or a single bivalent Ag-binding fragment [$F(ab')_2$], respectively (Fig. 1C). Complementary aspects of Ig structure are described in chapter A3.

Ig molecules are classified into five ISOTYPES based on the structure of their heavy chain C-terminal regions; IgM, IgD, IgG, IgE and IgA (Fig. 2). Each of the Ig ISOTYPES has distinct characteristics and functions within the overall humoral immune response (see below for details). The process by which different Ig ISOTYPES can be produced by daughter cells from the same activated B cell is called Ab isotype class switch (Box 3 and chapter A3).

Immunoglobulins of class G

IgG is the most abundant Ig in human plasma, where it accounts for approximately 75% of all Ig molecules. The class switch to IgG often occurs in response to interferon-γ derived from Th1 polarised T cells, which are stimulated by viral and bacterial structures. IgG is found in both intra- and extravascular pools in monomeric form. IgG molecules are involved pre-

Box 2

Ig molecules have two functions; the first is Ag recognition by the Fab portion and the second is mediation of an effector response by the Fc portion.

Box 3

Naïve B cells express membrane-bound IgD and IgM with identical variable regions. Following activation, isotype class switching allows the daughter cells of activated B cells to produce different Ig isotypes depending on the effector function required, i.e. the Ig produced has the same variable region as the parent Ig but a different constant region.

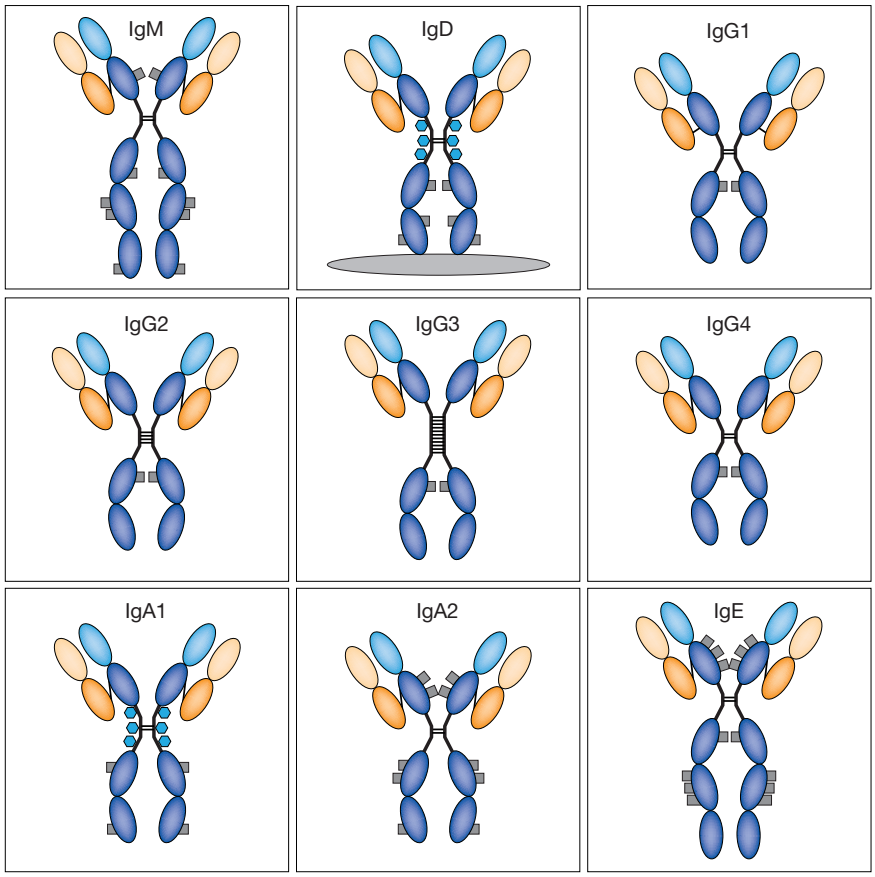

FIGURE 2. STRUCTURE OF ALL IG ISOTYPES AND SUBCLASSES
N-linked carbohydrates are shown as grey rectangles; clusters of O-linked carbohydrates are shown as blue hexagonals. Please note that the number of O-linked carbohydrates in a cluster varies.

dominantly in the secondary Ab response, namely the response observed after repeated exposure to an Ag. IgG molecules bind to many types of pathogens including viruses, bacteria and fungi. Once bound, they mediate the inactivation or removal of these pathogens *via* mechanisms such as opsonisation, leading to complement activation (C1q binding) and PHAGOCYTOSIS as well as toxin neutralisation (see chapter A6).

IgG molecules are also involved in Ab-dependent cellular CYTOTOXICITY (ADCC) by binding to NATURAL KILLER CELLS and GRANULOCYTES *via* the Fcγ RECEPTOR (FcγR; Tab. 2), in particular FcγRI, FcγRIIA and FcγRIII. IgG can also bind to FcγR on B cells and DENDRITIC CELLS, especially FcγRIIB, which is the only FcγR that mediates inhibitory signalling and thus fulfils important functions in down-regulation of ongoing immune responses.

The Fc portion of IgG can also bind to the neonatal Fc RECEPTOR (FcRn), which is involved in the transfer of IgG across the placenta to the foetus, providing humoral immunity before birth and during the first 6 months of life; IgG is the only Ig to pass through the placenta (Tab. 2).

Table 2. Biological functions of human Ig classes (isotypes) and subclasses

Immunoglobulin	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
Adult serum level range, g/L (mean g/L)	4.9–11.4 (6.98)	1.5–6.4 (3.8)	0.20–1.10 (0.51)	0.08–1.40 (0.56)	0.7–1.7	1.5–2.6 (a)		0.04	0.0003
Proportion of total IgG (%)	43–75	16–48	1.7–7.5	0.8–11.7	–	–		–	–
Half–life (days)	21	21	7.5–9	21	5	6 (a)		3	2.5
% intravascular	45–52 (all IgG)				76	40–42 (a)		75	–
Placental transfer	++	+	++	++	–	–	–	–	–
Antibody response to:									
Proteins	++	+/–	++	+/–					
Polysaccharides	+	++	(–)	(–)					
Allergens	+	(–)	(–)	++					
Complement activation:									
Classical pathway activation (C1q binding)	++	+	+++	–	+++	–	–	–	–
Alternative pathway activation	–	–	–	–	–	+	+	–	–
Binding to Fcγ receptors:									
FcγRI (CD64): monocytes, macrophages, neutrophils, eosinophils, dendritic cells	++	–	+++	+	–	–	–	–	–
FcγRIIA (CD32): monocytes, macrophages, neutrophils, eosinophils, platelets, B cells, dendritic cells, endothelial cells	++	(b)	++	–	–	–	–	–	–
FcγRIIA-H131	++	+++	+++	–	–	–	–	–	–
FcγRIIA-R131	++	–	++	–	–	–	–	–	–
FcγRIIB (CD32): B cells, mast cells, dendritic cells	++	–	++	(+)	–	–	–	–	–
FcγRIII (CD16)					–	–	–	–	–
FcγRIIIA: NK cells eosinophils, macrophages, subsets of T cells, mast cells (c)	++	–	+++	–	–	–	–	–	–

Table 2. *(continued)*

Immunoglobulin	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
FcγRIIIB: neutrophils									
FcγRIIIB-NA1	+++	–	+++	–	–	–	–	–	–
FcγRIIIB-NA2	++	–	++	–	–	–	–	–	–
FcεRI: mast cells, eosinophils, basophils	–	–	–	–	–	–	–	–	+++
FcεRII (CD23): B cells, T cells, follicular dendritic cells, eosinophils, platelets, Langerhans' cells	–	–	–	–	–	–	–	–	++
FcαR (CD89): monocytes, neutrophils, eosinophils, T cells, B cells	–	–	–	–	–	++	++	–	–
FcμR: T cells, macrophages	–	–	–	–	+	–	–	–	–
FcδR: T cells, B cells	–	–	–	–	–	+	–	+	–
Poly Ig receptor, mucosal transport	–	–	–	–	+	+	+	–	–
Binding to:									
Staphylococcus protein A	++	++	–	+	–	–	–	–	–
Streptococcus protein G	++	++	++	++	–	–	–		–
Virus neutralisation	+	+	+	+	+	+	+	–	–
Functions	Opsonisation, complement activation, ADCC, neonatal immunity, feedback inhibition of B cells				Naïve B cell Ag receptor, complement activation	Mucosal immunity In plasma: anti-inflammatory		Naïve B cell Ag receptor	Defence against helminth parasites; immediate hypersensitivity

ADCC, Ab-dependent cellular cytotoxicity; NA, neutrophil antigen; NK, natural killer; Staph., Staphylococcus, Strept., Streptococcus. a) refers to plasma IgA, IgA1 is the predominant form, making up 90% of the total IgA; b) FcγRII allotype-dependent; c) Polymorphisms in FcRγIIIA gene Phe158Val affect IgG binding and functional activities. Receptors for IgG: CD64, high affinity; CD32 and CD16 are both of low affinity. From [102, 103] and Immunobiology (Janeway) with modifications.

IgG molecules are further divided into four subclasses based on differences in amino acid sequence, especially in the hinge region (Fig. 2). Numbered based on their relative abundance in human serum, IgG1 accounts for 60–70% of the total IgG, IgG2 14–20%, IgG3 4–8% and IgG4 2–6%. The variations in the hinge region of the IgG subclasses result in differences in their flexibility; the flexibility decreases in the order IgG3, IgG1, IgG4 and IgG2. These subclasses also differ in their ability to bind effector molecules. For example, IgG3 binds complement factor C1q with high AFFINITY, IgG1 with intermediate AFFINITY and IgG2 with low AFFINITY, while IgG4 is unable to activate complement (Tab. 2). Similarly, variation in the AFFINITY of binding to FcγRI, IIA, IIB, IIIA or IIIB accounts for the differences in FcγR-mediated effector functions of IgG subclasses.

Immunoglobulins of class M

IgM molecules make up 10% of the total serum Ig content. They are confined predominantly to the intravascular pool and are part of the primary, antigen-specific, humoral immune response; phylogenetically and ontogenetically they are the earliest Ab molecules. IgM has an extra constant domain in the heavy chain (C_H4) (Fig. 2) and when secreted, it exists predominantly as a pentamer joined by the J chain and arranged into a planar structure (Fig. 3). Electron microscope studies showed a star-like structure, which, when bound to Ag, assumes a crab-like conformation. Occasionally, IgM can also be found in a hexameric form (Fig. 3).

IgM is a very potent activator of the classical complement pathway by its efficient binding of C1q through the C_H3 domain. Indeed, IgM is unable to enhance the Ab response without a functioning COMPLEMENT SYSTEM [9].

Naturally occurring ANTIBODIES, termed NATURAL ANTIBODIES (NAb), utilise germline-encoded genes in the variable region, are generated in the newborn in the absence of external Ag stimulation and are directed mainly against self and altered self components [10]. NAb are often of IgM isotype and fulfil important functions in tissue homeostasis (CLEARANCE of oxidatively damaged structures, CLEARANCE of apoptotic cells, CLEARANCE of intracellular proteins released from necrotic cells) and tumour surveillance (recognition of newly emerging carbohydrate, glycolipid or glycoprotein pattern on malignant cells). Other actions of IgM NAb molecules include proteolysis and modulation of B and T cell immune responses, as well as promotion of diseases arising from a sudden impact, such as in infarction or systemic inflammatory response syndrome [11].

Immunoglobulin of class A

Serum IgA molecules are predominantly present as monomers and are produced by BONE MARROW plasma cells. They are the second most common Ig after IgG, making up 15–20% of the total serum Ig. Typically, IgA CLASS SWITCHING is induced by transforming growth factor-β or by INTERLEUKIN (IL)-5. IgA is also found as a dimer, synthesised by mucosal plasma cells of the intestinal lamina propria, the upper respiratory tract or the urogenital tract. Secretory IgA (SIgA) consists of an IgA dimer joined by one J chain, and a secretory component of 70 kDa (Fig. 3). To form SIgA, dimeric IgA released from mucosal plasma cells binds to the polymeric Ig RECEPTORS (pIgR) expressed on the basolateral side of mucosal epithelial cells. The pIgR-IgA complex is transported across the mucosal epithelial cells and when released into the secretions at the apical side of the epithelial cells, the pIgR is cleaved and one part, the secretory component, remains covalently bound with the IgA dimer. Association with the secretory component protects IgA from proteolytic degradation. SIgA is the predominant Ig in seromucous secretions such as saliva, tracheobronchial secretions, colostrum, milk, tear fluid, intestinal secretions and genito-urinary secretions. It is the most prominent Ig produced at mucosal linings (and thus in the human body); approximately 3–5 g IgA is secreted daily into the intestinal lumen.

There are two subclasses of IgA, IgA_1 and IgA_2 (Fig. 2). These two subclasses differ in their hinge region. While IgA_1 has a 19-amino acid hinge region, IgA_2 has only 6 amino acids. In serum, IgA_1 is the predominant form, making up 90% of the total IgA, whereas SIgA consists mainly of IgA_2.

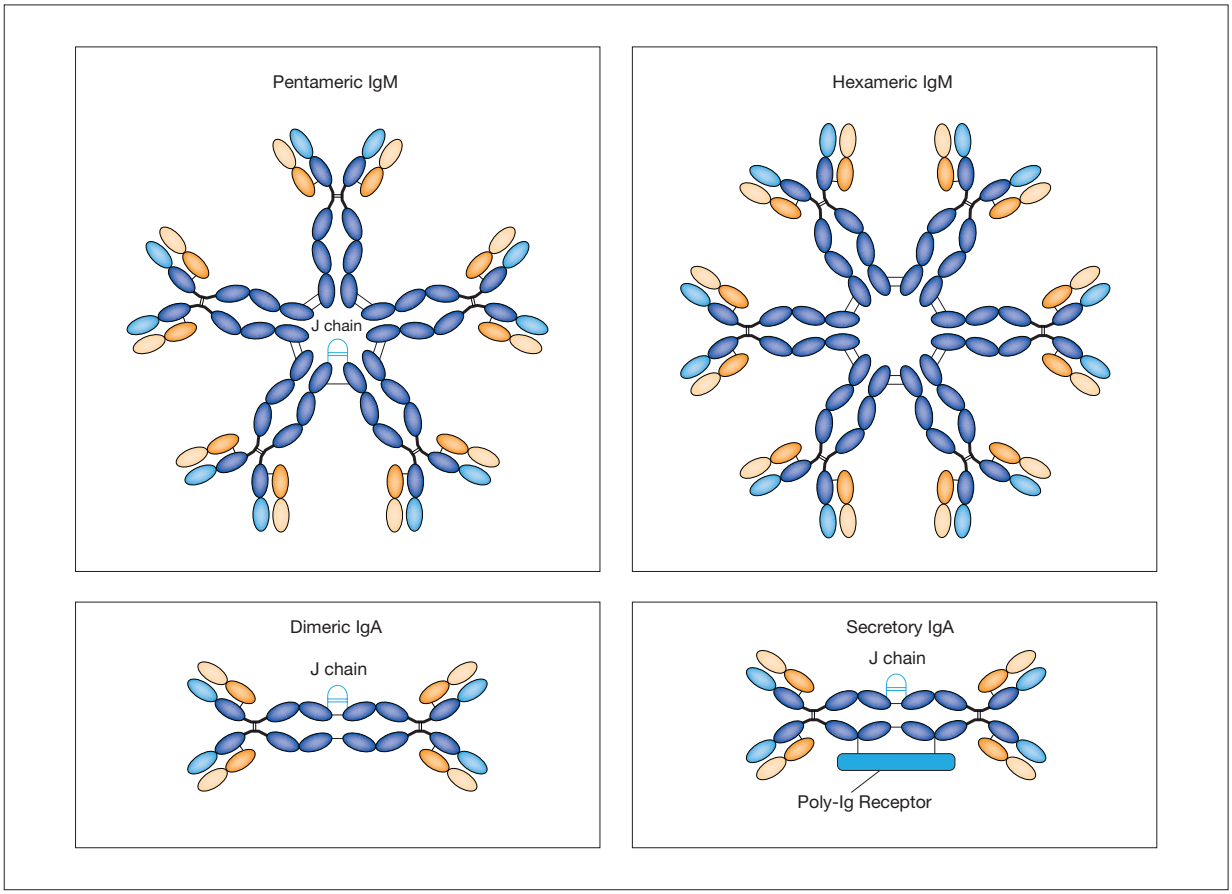

FIGURE 3. STRUCTURES OF POLYMERIC IGM AND IGA

Binding of monomeric serum IgA to the FcαRI present on MONOCYTES, MACROPHAGES, NEUTROPHILS and EOSINOPHILS, induces inhibitory signalling pathways [12]. Thus serum IgA serves an important anti-inflammatory function in the systemic circulation. In aggregated form, serum IgA can activate the LECTIN PATHWAY of the COMPLEMENT SYSTEM as a result of binding to the carbohydrate recognition domain of MANNAN-BINDING LECTIN (MBL) [13].

SIgA is present as high- and low-AFFINITY Ab; high-AFFINITY SIgA helps defend mucous membranes of the intestine and nose against viral or bacterial infections and takes part in the neutralisation of bacterial toxins, while low-AFFINITY SIgA has a more 'homeostatic' function in shaping and controlling the commensal MICROBIOTA in the intestinal lumen by 'immune exclusion' [12]. SIgA can neither bind nor activate complement.

Immunoglobulin of class D

IgD is present at very low concentrations in serum; it accounts for less than 1% of the total serum Ig (Fig. 2). It is mostly found as a membrane-bound monomer, co-expressed with IgM, on naïve B cells. Upon activation of naïve B cells with specific Ag, class switch RECOMBINATION occurs and the expression of IgD is lost. Recently, a potential function of secreted IgD in anti-bacterial immunity of the respiratory tract has been described [14, 15].

Immunoglobulin of class E

IgE is found only in trace amounts in human serum. Class switch to IgE production is classically induced by IL-4 derived from Th2 polarised T cells, but alternative pathways have also been described. Similar to IgM, IgE has an extra constant domain in the heavy chain (C_H4) (Fig. 2). It is found predominantly bound to the high-affinity Fcε receptor (FcεRI) on basophils and mast cells even prior to the interaction with its cognate Ag. When Ag/allergen binds to the IgE on mast cells and basophils it causes the aggregation of the Fcε receptors and subsequent degranulation of the cell, with the release of vasoactive and chemotactic mediators. This results in allergic reactions such as hay fever, extrinsic asthma and the Prausnitz-Küstner skin reaction; in severe cases Anaphylaxis can even be life-threatening. As well as its role in atopic allergy, IgE is important in protection against helminths and parasites. Indeed, elevation of IgE levels can be used as a diagnostic tool for parasitic infections. Circulating IgE cannot activate complement *via* the classical pathway (Tab. 2).

IgE can bind to two Fc receptors, namely FcεRI and FcεRII. IgE is mostly bound to the FcεRI; the FcεRI binding site on IgE is located at the interface between C_H2 and C_H3. The IgE binding affinity for the FcεRII, which is an Fc receptor found on monocytes, B cells and platelets, is lower than for FcεRI.

Glycosylation of immunoglobulins

Glycosylation of Ig has a number of important roles including the maintenance of structure and stability, receptor binding, Fc effector function, intracellular transport, secretion and clearance, expansion of the Ab repertoire, and cross-talk between the innate and adaptive immune pathways. The five classes of Ig are highly diverse in terms of the location and number of conserved *N*-linked glycosylation sites situated on the Fc and Fab portions (Figs. 1A and 2) (see Box 4) [16]. Abnormalities in the glycosylation profiles of Ig molecules have been linked to certain diseases. For example, increases in agalactosyl glycoforms of IgG have been isolated from patients with rheumatoid arthritis; the pathogenesis of IgA nephropathy is influenced by abnormal IgA1 *O*-glycosylation and hence reduced IgA clearance; the glycosylation of all glycoproteins is also affected in congenital disorders of glycosylation and abnormal IgG glycoforms are used in the diagnosis of these disorders [16].

IgG glycosylation

All IgG molecules have a single conserved *N*-linked glycosylation site, Asn297, in the Fc region, which is important in maintaining Fc effector function (Figs. 1A and 2). The oligosaccharides present in this region are of the complex di-antennary type. Comprised of a core heptasaccharide, these oligosaccharides have the variable addition of fucose, galactose, bisecting *N*-acetylglucosamine and sialic acid. Sialylation tends to be present in less than 10% of IgG. Glycosylation is critical for effector functions mediated *via* the FcγR, the C1q component of complement and the MBL. For example, the absence of core fucose on the Fc glycans leads to increased binding to FcγRIIIA and enhanced ADCC [17]. Furthermore, terminal sialylation of the Fc glycans has been reported to be responsible for the anti-inflammatory properties of human IgG in mice [18]. In contrast, Fc glycosylation does not appear to play a role in FcRn interactions [17].

Box 4

Only 2–3% of the molecular weight of IgG is made up of glycosylation in the heavy chain, whereas IgM, IgD and IgE molecules are more highly glycosylated; 12–14% of the molecular weight is accounted for by glycosylation of the heavy chain.

In addition to Fc glycosylation, approximately 20–30% of normal plasma IgG is glycosylated in the FAB REGION. In contrast to the Fc oligosaccharides, Fab oligosaccharides are bisected, extensively galactosylated and substantially sialylated. This difference may be due to a lack of accessibility of the Fc portion to specific transferases [19].

Various factors influence the glycosylation of IgG, including age and sex. Age dependency is particularly clear; galactosylation and sialylation increases up to the age of 25 years and then decreases again throughout life. Moreover, during pregnancy there is a temporary increase in IgG galactosylation.

Glycosylation of other Ig molecules

IgA molecules possess two conserved *N*-glycosylation sites in the heavy chains, one in the C_H2 region and one in the tailpiece of the Fc region (Fig. 2). In addition, there are two to three further *N*-glycosylation sites in IgA_2, up to five *O*-glycosylation sites in the hinge region of IgA_1, seven *N*-glycosylation sites in the secretory component and a further one in the J chain. In contrast to IgG, over 90% of IgA molecules are sialylated [20].

The μ-chain of IgM molecules has five *N*-linked glycosylation sites; three are of the complex type and two of the oligomannose type. These contain predominantly monosialylated, bi-antennary structures. In addition, there is one *N*-linked glycosylation site in the J chain containing mainly sialylated bi-antennary glycans. Circulating predominantly as pentamers, the glycan EPITOPES are thought to allow IgM to agglutinate microorganisms in the serum [16].

IgE is the most heavily glycosylated Ig. It contains seven *N*-linked glycosylation sites in the ε-chain. These *N*-linked glycans result in a reduction in the flexibility of the IgE molecules, which is thought to control Ag binding and prevent unwanted and potentially fatal immune responses through inappropriate Ag binding [16].

IgD has three *N*-linked glycosylation sites in the δ-chain and site-directed mutagenesis has shown that the presence of oligomannose glycans at Asn354 is of vital importance for the production of IgD. Absence of this site results in incomplete assembly and lack of secretion [16].

Pharmacokinetics of immunoglobulins

Catabolism provides the main route of Ig elimination [21]. Due to its large size, very little Ig is excreted in the urine and only small amounts have been identified in the bile. It is assumed that approximately one-third of Ig is broken down in the liver, with another third in the intestines. The cellular compartments in close proximity to the blood and lymph such as the endothelial cells constitute other sites important in Ig catabolism. There are differences in sites of catabolism between Ig classes with IgA, particularly SIgA, being different from other classes as it is present in high amounts in body excretions such as intestinal fluid and saliva with a resulting high turnover.

Pharmacokinetics of IgG

In pharmacokinetic studies with IVIG administration, a two-compartment model has been proposed to describe the time course of IgG serum concentrations (Fig. 4A) [22, 23]. After the initial rapid rise in serum IgG concentration a comparably rapid decrease follows (Fig. 4B). This early (or α) phase, which in humans roughly occurs during the first 5 days after infusion, is the result of both catabolism of IgG and distribution between the intravascular and extravascular spaces. The following β phase shows a slower decrease in IgG serum concentration, which is caused by the catabolism of IgG and thus determines the serum half-life of IgG. This general model has been confirmed using various IVIG preparations [23, 24]. A high inter-individual variability of pharmacokinetic parameters was found, which is not unexpected considering the complex mechanisms of catabolism and distribution of IgG [24, 25].

The use of radioiodinated IgG molecules has shown that IgG, except for IgG3, can survive longer in the blood than any other serum proteins, with a mean half-time of survival of about 3 weeks (Tab. 2)

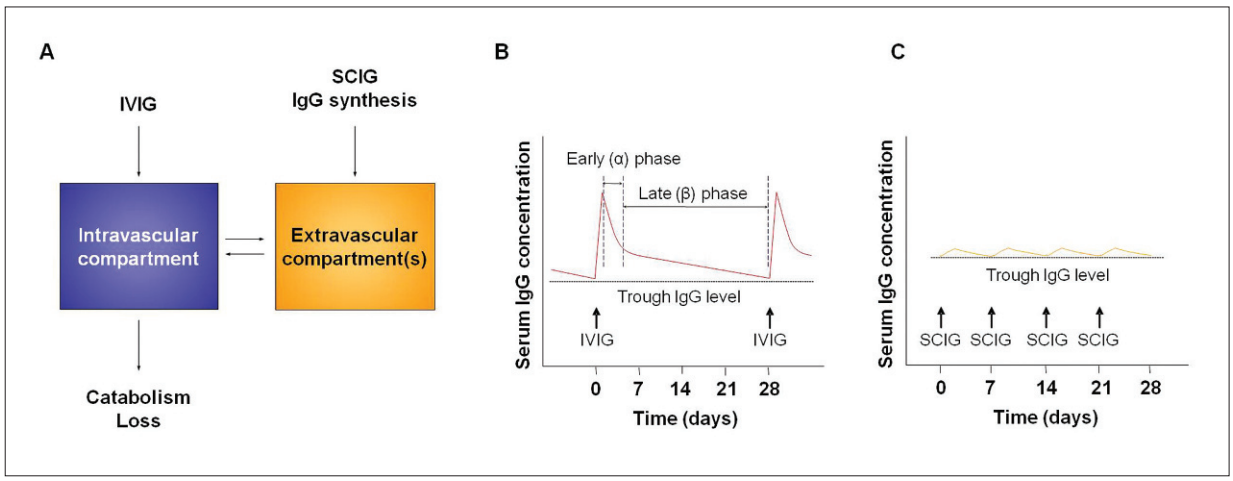

FIGURE 4. TWO-COMPARTMENT MODEL OF IgG PHARMACOKINETICS

(A) IgG is in equilibrium between the vascular space and extravascular areas. Synthesised in the bone marrow, IgG diffuses into the lymph and then into the blood. IVIG enters the intravascular space directly, whereas SCIG is absorbed from the subcutaneous tissue. The catabolism of IgG occurs in the vascular endothelium, liver and other areas. IgG may also be lost from the vascular space by other mechanisms such as protein loss in the intestines or urinary tract. (B) Serum IgG concentration over time after IVIG administration. The initial early α phase is due to the passage of IgG from the vasculature to the lymph and extracellular fluid compartments in addition to catabolism. The late β phase is due to IgG catabolism. From [22], with modifications. (C) Serum IgG concentration over time after SCIG administration. Fluctuations in IgG levels are essentially absent with SCIG administration and mean trough levels are higher compared with IVIG treatments.

for the subclasses IgG1, IgG2 and IgG4, while IgG3 has a shorter half-life of 7.5–9 days [21, 22].

The IgG half-life is significantly longer than the half-lives of other Ig classes due to its binding to the FcRn (except for IgG3). Initially identified for its role in the transfer of IgG from the mother to the foetus across the placenta, the FcRn has also been found to prolong the half-life of IgG (and albumin) throughout life. Thus, as it is expressed on a multitude of cells and tissues such as endothelium, interstitium, kidneys, lungs and blood-brain barrier, it helps to maintain high IgG concentrations in the circulation at a comparably low synthesis rate [26, 27].

The proposed cycle of IgG protection through FcRn binding is summarised in Figure 5 (see also Box 5). In brief, IgG is taken up by endosomes in cells such as vascular endothelial cells and muscle cells. As the pH of the endosome decreases, the AFFINITY of the IgG molecules for the FcRn increases and they bind to the RECEPTOR. After fusion with the lysosomes, unbound IgG is degraded, while the FcRn-bound IgG does not enter the lysosome, remains intact, is subsequently returned to the plasma or interstitial fluid and finally released from the FcRn as the pH rises to neutral. This proposed pathway may explain the observation that as IgG serum concentrations rise, the IgG half-life decreases because increasing concentrations of IgG result in a saturation of the FcRn and hence more IgG molecules enter the lysosome and are catabolised [26]. The importance of the level of saturation of FcRn on the length of IgG half-lives has also been shown in a mouse model of AUTOIMMUNE DISEASE [28]. Treatment of mice with high-dose IVIG, resulting in high IgG serum concentration, reduced the half-life and thus the serum concentration of the monoclonal mouse IgG applied in constant amounts as a model autoantibody. This result provides evidence for one of several MECHANISMS OF

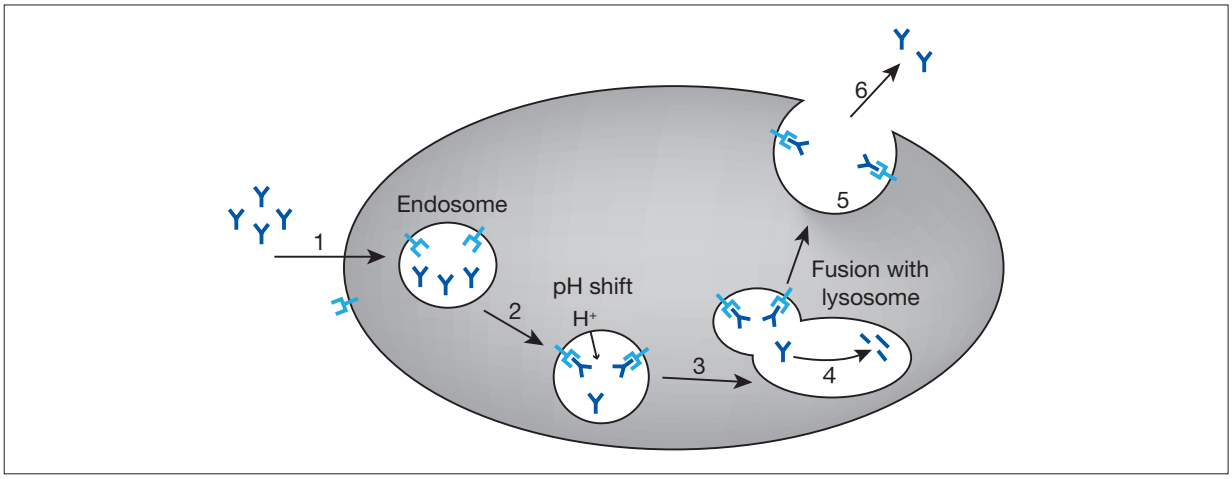

Figure 5. Proposed mechanism for IgG protection by FcRn
(1) IgG molecules enter cells (e.g. vascular endothelial cells, muscle cells, etc.) via pinocytosis (e.g. fluid-phase endocytosis); (2) H^+ enters the endosome, reducing pH, and IgG binds with FcRn; (3) the endosome fuses with the lysosome, but FcRn-bound IgG is not released to the lysosome; (4) unbound IgG is released into the lysosome and catabolised by proteases; (5) the endosome fuses with the plasma membrane, the FcRn-IgG complex is exposed to neutral pH; and (6) IgG is released into plasma (or interstitial fluid). From [104], with modifications.

action of high-dose IVIG treatment, i.e. the reduction of autoantibody serum concentrations in autoimmune disease. On the other hand, in diseases such as PID, treatment with human IgG generally results in lower than normal IgG serum concentrations with resulting serum half-lives longer than normal [25].

Plasma levels of IgG are affected by other factors besides FcRn. IgG synthesis can be inhibited by abnormal increases in IgG concentrations resulting in negative feedback inhibition. Differentiation of B lymphocytes to plasma cells and the expansion of the latter are inhibited. In contrast, a reduction in IgG concentration, for instance as a result of plasmapheresis, may result in an increase in Ig synthesis.

Immunoglobulin distribution

Close to half of the total body pool of IgG can be found in the intravascular space (Tab. 2) and the amount of IgG transferred from the intravascular pool to the extravascular space has been reported to be between 27% and 39% per day. As IgG does not cross the blood-brain barrier under normal conditions, IgG concentrations in the cerebrospinal fluid (CSF) are low, at around 25 mg/L (compared with about 10 g/L in serum). However, in certain neurological and inflammatory diseases such as bacterial meningitis, IgG can readily enter the CSF [27, 29] due to a transient opening of the blood-brain bar-

Box 5

IgG is the only isotype capable of binding to FcRn (except for IgG3). This protects IgG from proteolytic cleavage and results in the long serum half-life for IgG1, IgG2 and IgG4 of about 3 weeks, compared with 2.5–9 days for other isotypes (including IgG3).

rier. In these situations, FcRn is thought to play a role in removal of IgG from the CENTRAL NERVOUS SYSTEM (CNS). Such mechanisms are also discussed for diseases such as Alzheimer's disease.

IgG constitutes one of the immunoglobulin classes excreted into milk and colostrum in humans and rodents, with some species differences. An FcRn-dependent absorption of IgG from the gut to the blood of the newborn is observed in rodents, but only minimally in humans. In humans, IgG is transferred prenatally solely across the placenta. At term, three to five times higher serum IgG levels are found in the neonate than in the mother. In the neonate, serum concentrations of IgG1 and IgG4 are higher, IgG3 equal, and IgG2 lower than in the serum of the mother (see also Tab. 2) [30]. FcRn seems to be one of the RECEPTORS involved in placental IgG transport in humans.

The intravascular distribution of IgM and IgA differs from IgG. More than three-quarters (76%) of IgM can be found within the intravascular space. For IgA this proportion is lower, with only 40–42% being found intravascularly (Tab. 2).

Subcutaneous and oral administration of IgG

The s.c. administration of IgG has expanded with the prospect of increased convenience (home therapy), more sustained IgG levels and an improved safety profile. In contrast to i.v. treatment, after s.c. administration, IgG passes first into the lymphatic system and from there into the bloodstream [31]. However, direct comparisons of IgG BIOAVAILABILITY using the i.v. and s.c. routes are very difficult for a number of reasons. These include the difference in early distribution after s.c. and i.v. administration, the lower amount of IgG administered s.c. *versus* i.v., the different frequencies of administration and the sole measurement of IgG levels in serum, but not in other body fluids in most studies. As SCIG is administered at a higher frequency and the release from the s.c. depot into the intravascular space is slow with maximal plasma concentrations being reached 4–6 days after infusion [32], fluctuations in IgG levels are essentially absent with SCIG administration and mean trough levels are higher compared with IVIG treatments (Fig. 4B, C) [22].

Little is known about oral absorption of IgG. The acid conditions of the stomach provide a hostile environment for any protein. Nevertheless, upon oral administration of human IgG to PID patients experiencing rotavirus infections, about 50% of IgG was found in stool samples, approximately 40% of which was in a higher molecular form, indicating formation of immune complexes [33]. At the same time, free rotavirus protein decreased in the stools. Similar results were obtained using bovine IgG containing anti-*Clostridium difficile* ANTIBODIES [34, 35]. However, absorption from the gut into the blood is minimal in humans, either FcRn mediated as discussed earlier or by diffusion, due to the large molecular size and high polarity of IgG.

Pharmacokinetics of other isotypes

Just under half of all IgA molecules are found in the intravascular space. They have an average half-life of 6 days, which is shorter than that of IgG molecules (see Tab. 2) [21, 22, 36]. One of the main reasons for this shorter half-life is that IgA molecules do not bind to FcRn and are not protected from degradation [37]. However, at mucosal sites IgA molecules do bind to the polymeric Ig RECEPTOR (pIgR) (see above), which affords some protection from degradation.

Very little is known about the pharmacokinetics of the other Ig classes. In terms of distribution, unlike IgG, both IgM and IgD are found predominantly in the intravascular space (Tab. 2); for IgM this is due to its large molecular weight [21].

Immunoglobulin preparations for medical use

The history of Ig preparations

Preparations containing Ab for 'SERUM THERAPY' were introduced by Emil von Behring and Shibasaburo Kitasato more than 100 years ago [38]. The first 'antitoxins' were heterologous serum preparations from hyperimmunised animals with Ab SPECIFICITY to diphtheria and tetanus toxins. Unfortunately, the

initial success, particularly with the diphtheria antisera, was marred by the increasing incidence of side effects from the heterologous animal serum proteins, known as serum sickness. As a result, purer and therefore more compatible preparations were developed.

The initial Ig preparations derived from human plasma were for i.m. injection only. Disadvantages of the i.m. administration included pain at the injection site, limits to the volumes that could be administered, reduced BIOAVAILABILITY, and delays of at least 24 hours and up to several days before maximum serum levels were achieved. The i.m. preparations of Ig (IMIG) were not suitable for i.v. administration due to adverse reactions, which were often severe and even fatal. Such adverse reactions occurred in at least 15% of administrations and were attributed to the presence of aggregates in these preparations [6]. Ig aggregates can interact with the first component of the COMPLEMENT SYSTEM, C1q, thereby triggering the CLASSICAL PATHWAY of complement activation and causing the *in vivo* generation of ANAPHYLATOXINS (C3a, C5a). The presence of endogenous prekallikrein activator (PKA) activity, known to induce hypotension, further contributed to the adverse event profile of IMIG after i.v. administration. Together with the lack of appropriate stability, these factors made the initial IMIG preparations unsuitable for i.v. administration.

Initial IVIG preparations developed in the 1970s underwent plasma fractionation by cold ethanol precipitation followed by protease digestion, including trypsin, plasmin and pepsin. The resulting Ig preparation contained predominantly fragmented Ig including the bivalent Ab molecule, $F(ab')_2$ (Fig. 1C). However, these preparations contained no Fc-mediated activity, which is essential for the full spectrum of Ig activities. Moreover, although well tolerated, these preparations had a very short half-life of 24 hours or less, making them generally unsuitable for Ab replacement therapy.

Subsequent IVIG preparations were produced by cold ethanol precipitation followed by chemical modification, which led to a marked reduction in inherent complement binding [39]. Unfortunately, such chemical treatments led to reversible or irreversible modifications of the Ig molecule, which did not demonstrate fully active Fab and Fc functions. Although effective in the treatment of some diseases, they were not as functional as native, intact Ig. As a result, they became obsolete for all major markets.

Immunoglobulin formulations today

More recent developments in the production of IVIG rely on more gentle isolation and purification procedures [40]. Cold ethanol fractionation is followed by a series of individual purification procedures that may also be used in combination. Polymers such as polyethylene glycol, which support selective precipitation and stabilisation may also be used. State of the art production processes use cation and/or anion exchange chromatography for polishing the IgG, especially for the removal of Ig classes other than G (i.e. IgA, IgM, IgE). These techniques yield unaltered and functionally intact IgG with at least 96% IgG, a subclass distribution characteristic of human serum and a normal half-life of IgG (Box 6).

Virus inactivation and removal steps are integrated within the IVIG production process. More detail on this topic is given later in the chapter (section "Viral safety of IMMUNOGLOBULINS").

Lyophilised IVIG preparations offer excellent stability and have a shelf life of up to several years at room temperature. A disadvantage of lyophilised products is that they are less convenient because they require reconstitution prior to administration.

With this in mind, the latest preparations of IVIG have been formulated as a ready-to-use liquid. While

Box 6

Current production methods for IVIG generate non-cleaved, native and functionally intact IgG with an almost normal distribution of IgG subclasses and a physiological half-life.

the first liquid preparations of IVIG required refrigeration and had a limited shelf life, more recent formulations are stable at room temperature for extended periods. Studies have shown that by reducing the pH to moderately acidic and adding appropriate stabilisers to the formulation, the stability of the liquid IVIG can be maintained for up to 3 years [41, 42].

In liquid IVIG, isolated from pooled plasma of numerous donors, IDIOTYPE/anti-IDIOTYPE Ab dimers are formed [43, 44]. IDIOTYPE/anti-IDIOTYPE dimers form when the Ag-binding region of one IgG molecule binds to the variable region of another. High levels of dimeric IgG in IVIG have been described to be associated with adverse reactions such as headache, fever and flushing observed during i.v. infusion [45, 46]. Therefore, in liquid IVIG preparations the formation of IgG dimers has to be controlled to maintain good clinical tolerability.

Table 3 (see pp. 288/289) provides characteristics of a selection of currently available IVIG preparations in major markets.

For certain patients, such as children, pregnant women, and individuals with poor venous access, s.c. administration of Ig is more convenient [32]. Moreover, a number of validated health-related quality of life questionnaires have found that many patients prefer SCIG to IVIG [32, 47]. Although some of the current preparations of IVIG are suitable for s.c. administration, specific formulations of Ig for s.c. administration are being developed. So far, the FDA has approved the use of a few SCIG preparations. In Europe SCIG is more extensively used [48]. The newest SCIG preparation contains Ig concentrations as high as 20% (Tab. 3) [49].

The basic material for the production of polyvalent human Ig, polyvalent homologous Ig (or, according to pharmacopoeia terminology, 'normal' Ig), is a pool of plasma from thousands of healthy human donors. Special Ig may be isolated from the plasma of specifically immunised donors, from the plasma of convalescents or from screened plasma containing high titres of a desired SPECIFICITY. Most of the plasma obtained for the production of therapeutic Ig is obtained by plasmapheresis and is called 'source' plasma. Plasma from whole blood donations, termed 'recovered' plasma, is also used [49]. Despite the use of a large number of donors, Ig preparations still demonstrate a high degree of variability in SPECIFICITY and titre levels. Each Ig preparation is different; it represents the cumulative Ag experience of the donors and therefore depends on the donor population and also the period of time when the plasma was collected. For example, vaccination programmes have significantly affected titres of the pooled Ig preparations. As the measles disease triggers a higher Ab titre than the vaccine, mean measles titres have been declining since the introduction of a widely accepted vaccination programme, whereas mean titres to HEPATITIS B VIRUS have increased significantly since the mid nineties (after the introduction of the vaccine) [50].

Hyperimmune plasma from volunteers immunised with approved vaccines against diseases such as hepatitis B, varicella zoster, tetanus toxoid or rabies provides a source of pathogen-specific, high-titre therapeutic Ig [48]. This passive prophylaxis against certain infectious diseases is receiving increasing interest. While the use of animal sera and antitoxins often results in unwanted side effects due to the non-human origin [4], hyperimmune Ig from human sources are similar to regular IVIG but contain a high level of Ab against a specific Ag and are often useful in certain conditions where normal Ig would be of little benefit [48]. A good example is anti-RhD IgG prepared from the plasma of RhD-negative human donors who agree to be hyperimmunised with RhD-positive red blood cells. This preparation has been successfully used for decades to prevent RhD incompatibility between mother and child in the course of pregnancy or during delivery, or after mistransfusion of RhD-positive blood to RhD-negative recipients. Recently, a new production process has been developed that selectively concentrates anti-D IgG and provides higher yields of this hyperimmune Ig than conventional manufacturing processes for plasma-derived IgG [51].

Table 1 provides details of a selection of currently available hyperIg preparations.

Indications for immunoglobulins

When first introduced, IVIG was used as a replacement therapy for immunodeficiency diseases. Currently, IVIG is also used as a treatment for a number of autoimmune and inflammatory diseases (Box 7). IVIG is used at low doses when administered as a 'replacement' therapy (200–500 mg/kg) and at high doses when used as an 'immunomodulatory/anti-inflammatory' therapy (up to 2 g/kg).

Immunoglobulin replacement therapy in primary immunodeficiencies

IVIG is indicated as a replacement therapy in patients with primary immunodeficiency (PID) characterised by reduced or absent Ab production and recurrent infections [52–54]. More than 140 distinct, inherited PID disorders have been identified [55]. The most common ANTIBODY deficiency is common variable immunodeficiency (CVID) [56].

X-linked agammaglobulinaemia

In a rare X-linked genetic disorder, patients with X-linked agammaglobulinaemia experience repeated infections, particularly of the respiratory tract, paranasal sinuses, ears and meninges. Pneumococci or encapsulated *Haemophilus influenzae* are often the cause. Intestinal infections caused by bacteria such as *Campylobacter jejuni* are also common. In these patients, who have a deficit in Ab production, IVIG is of clear benefit, reducing both acute and chronic infections. Analyses of data in agammaglobulinaemic children have found that the number and severity of infections is inversely proportional to the dose of IVIG [53]. Recipients of stem cell transplants for SCID are often agammaglobulinaemic and also benefit from IVIG.

Hypogammaglobulinaemia

In hypogammaglobulinaemia, patients present with recurrent and often severe infections such as sinusitis, otitis media, pneumonia and meningitis. The infections may also involve the bones and joints and complications such as chronic asymmetric polyarthritis are not uncommon. Hypogammaglobulinaemia is a characteristic of CVID and studies in these patients have demonstrated the benefits of IVIG, with a reduction in the incidence of infections compared with the pre-treatment rates. CVID patients are particularly at risk of chronic, subclinical lung and pulmonary infections. IVIG can reduce the incidence of pneumonia and also prevent the progression of lung disease in these patients [53].

Regular treatment with IVIG is also of benefit to patients with hyper-IgM syndromes, characterised by hypogammaglobulinaemia and severe impairment of specific Ab production. Studies found that meningitis was eradicated in the patients tested and the incidence of pneumonia was markedly reduced [53].

Other primary immune deficiencies

Other well-defined immunodeficiency syndromes include hyper-IgE syndrome, in which patients demonstrate normal levels of IgG, IgM and IgA but have poor Ab-mediated responses, and Wiskott-Aldrich syndrome, which is characterised by normal IgG levels but abnormal Ab responses [53]. In addition, conditions caused by DNA repair defects, such as ataxia-telangiectasia, and thymic defects, as well as immune-osseous dysplasias, chronic mucocutaneous candidiasis, hepatic veno-occlusive disease

Box 7

IVIG is indicated in the treatment of PID as well as some secondary immunodeficiencies. In addition, IVIG has been used to successfully treat a number of autoimmune diseases including ITP, Kawasaki disease, GBS and CIDP. Experimental indications for IVIG include Alzheimer's disease.

Table 3. Various i.v. and s.c. immunoglobulin preparations

Product	Manufacturing process	Viral safety	Excipients	Protein	Storage, physical state	Route of administration	Manufacturer
Flebogamma DIF	Ethanol, PEG, IEX	S/D, past, virus filtration	Sorbitol, PEG, NaCl	5%	2–25°C, liquid	i.v.	Instituto Grifols
Gammagard Liquid (US) Kiovig (EU)	Ethanol, IEX	S/D, virus filtration, low pH	Glycine	10%	2–8°C, liquid	i.v.	Baxter
Gammagard SD	Ethanol, AIX	Ethanol-prec, S/D	Albumin, glycine, glucose, PEG, P80	5%, 10%	0–25°C, lyo	i.v.	Baxter
Gammanorm	Ethanol	S/D	Glycine, NaCl, NaAc	16.5%	2–8°C, liquid	s.c.	Octapharma
Gammaplex	Ethanol, IEX	S/D, low pH, virus filter	Sorbitol, glycine, NaAc, NaCl, P80	5%	2–25°C, liquid	i.v.	Bio Products Laboratory
Gamunex	Ethanol, caprylate, AIX	Caprylate, low pH, chromatography	Glycine	10%	2–8°C, liquid	i.v.	Talecris
Hizentra	Ethanol, AIX	Low pH, caprylate fractionation/depth filtration, virus filtration	Proline	20%	2–25°C, liquid	s.c.	CSL Behring
Intragam P	IEX	Past, low pH	Maltose	6%	2–8°C, liquid	i.v.	CSL
Intratect	CIX	S/D	Glycine	5%	2–25°C, liquid	i.v.	Biotest
Octagam	Ethanol, chromatography	S/D, low pH	Maltose	5%, 10%	2–25°C, liquid	i.v.	Octapharma
Omrigam	Ethanol	S/D, virus filtration	Maltose	5%	2–25°C, liquid	i.v.	Omrix
Privigen	Ethanol, AIX	Caprylate fractionation/depth filtration, low pH, virus filtration	Proline	10%	2–25°C	i.v.	CSL Behring
Sandoglobulin, Carimune NF	Ethanol	Low pH, pepsin, virus filtration	Sucrose	6%	2–30°C, lyo	i.v.	CSL Behring

Table 3. *(continued)*

Product	Manufacturing process	Viral safety	Excipients	Protein	Storage, physical state	Route of administration	Manufacturer
Subcuvia	N/A	S/D	Glycine, NaCl	16%	2–8°, liquid	s.c., i.m.	Baxter
Subgam	N/A	N/A	Glycine, NaAc, NaCl, P80	16%	2–8°C	s.c.	BioProducts Laboratory
Vivaglobin	Ethanol	Past, Ethanol-prec	Glycine, NaCl	16%	2–8°C, liquid	s.c.	CSL Behring

AIX, anion-exchange chromatography; CIX, cation-exchange chromatography; IEX, ion-exchange chromatography; N/A, no information available; PEG, polyethylene glycol; P80, polysorbate 80; S/D, solvent/detergent treatment; NaAc, sodium acetate; past, pasteurization; prec, precipitation

with immunodeficiency and Hoyeraal-Hreidarsson syndrome are also becoming increasingly well characterised [57]. Depending on the cause, symptoms of these conditions can include recurrent staphylococcal infections, eczema-like skin rashes and severe lung infections. Patients with these conditions benefit from IVIG treatment.

Immunoglobulin replacement therapy in secondary immunodeficiencies

Impairment of the IMMUNE SYSTEM as a result of disease, or secondary immunodeficiency, is not uncommon and IVIG treatment in these patients is often indicated. For example, patients with B cell malignancies and B cell chronic lymphocytic leukaemia (CLL), paediatric patients with HUMAN IMMUNODEFICIENCY VIRUS (HIV), and patients receiving BONE MARROW transplants have all been found to benefit from IVIG. Improvements in the incidence of infections, complications and quality of life are just some of the positive effects of IVIG in these patient groups [53].

Immunoglobulins and immunomodulation in autoimmune diseases

Immune thrombocytopenia

ITP is an AUTOIMMUNE DISEASE characterised by low platelet counts (less than 30×10^9/L) and mild to life-threatening mucocutaneous bleeding [58]. Many patients have no symptoms from ITP. Others present with bleeding. The hallmark presentation of ITP is mucocutaneous bleeding that manifest as purpura (petechiae, ecchymosis), epitaxis, menorrhagia, or oral mucosal, gastrointestinal, or intracranial haemorrhage. The rate of fatal bleeding is estimated at 0.02 to 0.04 cases per patient year, with the highest rate (0.13 cases per patient year) among patients older than 60 years.

Paediatric ITP is most commonly considered to be the acute form of ITP. Chronic ITP is a disease seen primarily in adults.

The standard of practice is to initiate treatment of ITP with oral prednisone or prednisolone at a dose of 1 mg/kg per day as suggested by both the American Society of Hematology (ASH) and the British Committee for Standards in Haematology (BSCH) guidelines [59, 60]. IVIG is suggested as a second line of treatment in these patients.

IVIG is likely to have multiple effects in patients with ITP. The blocking of the Fc RECEPTOR is thought to play a key role in the successful treatment of ITP with IVIG, with effects on B cells and Ab production, T cells and cell growth; the anti-inflammatory effects of IVIG are also thought to be important [52, 53, 61].

Recently, thrombopoietin (TPO) RECEPTOR agonists have been approved for the treatment of thrombocytopenia in patients with chronic immune thrombocytopenia (ITP) who have had an insufficient response to corticosteroids, IMMUNOGLOBULINS, or splenectomy. TPO RECEPTOR agonists initiate signalling cascades by binding to the transmembrane domain of the human TPO RECEPTOR, thereby inducing proliferation and differentiation of megakaryocytes.

Kawasaki disease

Kawasaki disease is an acute systemic INFLAMMATION of blood vessels and affects predominantly children under 5 years of age. It is complicated by coronary artery aneurysm in up to 25% of affected children.

High-dose IVIG, 2 g/kg over 8–12 hours in combination with acetyl salicylic acid (aspirin), is a well-established standard of care in the initial treatment of children. IVIG has been demonstrated in multiple randomised trials to reduce the prevalence of coronary aneurysm approximately fivefold when administered in the first 10 days, and ideally in the first 7 days, of illness [62–64].

The mechanism of action of IVIG in treating Kawasaki disease is unknown. IVIG appears to have generalised anti-inflammatory effects. The possible MECHANISMS OF ACTION include modulation of cytokine production, neutralisation of bacterial superANTIGENS or other aetiological agents, augmentation of T cell suppressor activity, suppression of ANTIBODY synthesis, and provision of anti-idiotypic Ab [65].

Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy

GBS is an acute demyelinating polyneuropathy characterised by acute (within 1 week) or subacute (within 4 weeks) ascending motor weakness, areflexia, and mild or moderate sensory abnormalities. It is a disease of all ages and occurs sporadically, although occasionally outbreaks have been noted. GBS is not one syndrome, but several syndromes, reflecting the varying degree of involvement of the motor or sensory nerve fibres and the myelin sheath and axon. These include acute inflammatory demyelinating polyneuropathy (AIDP), acute motor neuropathy (AMAN) and Miller-Fischer syndrome.

On the basis of two controlled clinical studies using IVIG *versus* plasmapheresis, IVIG, given at 2 g/kg over 2–5 days, has been shown to be equally effective as plasmapheresis, but no benefit was found when the two procedures were combined [66].

A Cochrane review of IVIG treatment in GBS, which included a meta-analysis of five trials involving 537 participants, concluded that IVIG can hasten the recovery of this syndrome to the same extent as plasma exchange [66]. The decision as to which treatment to use first, IVIG or plasmapheresis, is governed by circumstances, availability of the treatment modality, experience, age of the patients, and other associated conditions. Because IVIG is easy to administer and more readily available, and because time to initiate treatment is essential, IVIG has become the therapeutic choice worldwide.

Chronic inflammatory demyelinating polyneuropathy (CIDP) is the most common form of the autoimmune peripheral neuropathies. It can be considered as the chronic counterpart of GBS because of their various clinical, electrophysiological, histological, and laboratory similarities. CIDP differs from GBS predominantly by its speed of progression, mode of evolution, prognosis and the responsiveness to steroids.

CIDP presents with a slowly progressive weakness and paraesthesias that evolve over weeks or months. The weakness affects both distal and proximal muscles, and it follows a progressive or a relapsing-remitting course. Although CIDP is defined as an inflammatory polyneuropathy, there are only minimal signs of T cell infiltrates but many activated MACROPHAGES. Complement-fixing IgG and IgM are deposited on the patient's myelin sheath, indicative of myelin Ab. Ab to glycolipids LM1, GM1, or GD1b are also seen in some CIDP patients, but less frequently than in GBS patients.

In several clinical studies, IVIG has been effective in the majority of patients with CIDP. IVIG can be used effectively as a first-line therapy to avoid steroid-related side effects. A new randomised trial, the largest ever conducted *versus* placebo, demonstrated that IVIG is not only effective compared with placebo, but it had a long-term benefit. When given at 1 g/kg every 3 weeks for up to 1 year as a maintenance therapy, IVIG prevented relapses and axonal loss [67]. In addition, CIDP is classically a steroid-responsive polyneuropathy. The EFFICACY of steroids has been proven in a controlled study [68].

Multifocal motor neuropathy

Multifocal motor neuropathy (MMN) is a rare disease of the PERIPHERAL NERVOUS SYSTEM. MMN presents with progressive asymmetrical muscle weakness in one or more limbs. Although the pathophysiology of MMN is far from being understood, the presence of Ab directed against the ganglioside GM1 in nearly 50% of patients and the good response to immunomodulatory treatment, point to an immune-mediated mechanism.

MMN responds very well and essentially only to high-dose IVIG, which is the treatment of choice based on controlled clinical trials.

In a Cochrane review of four randomised studies including 34 patients, the authors concluded that IVIG does improve muscle strength with a possible improvement in disability [69]. Improvements in conduction block have also been reported with IVIG [70]. A recent retrospective study found obvious short-term benefits for MMN patients following IVIG treatment, although longer-term benefits were less well-defined in the 40 patients included in the study [71]. Recent studies have looked at the feasibility and safety of using SCIG in place of IVIG in MMN patients. SCIG was found to be as safe and effective as IVIG and may offer an alternative maintenance therapy in some patients [72, 73].

Myasthenia gravis

In most cases, the first symptom of myasthenia gravis is a weakness of the eye muscles. Other cases may present with difficulty swallowing or slurred speech. The degree of muscle weakness can vary significantly from case to case. Some may only involve the eye muscles, while others may result in more widespread muscle weakness.

Thymectomy is the most common treatment in most cases. In patients unsuitable for surgery or who have remained symptomatic following surgery, steroids are the most widely used immunosuppressive treatment. Other treatment options include cholinesterase inhibitors and plasma exchange. IVIG use in myasthenia gravis patients has been found to be comparable to plasma exchange, decreasing myasthenia gravis clinical scores [74, 75]. IVIG was generally better tolerated than plasma exchange [74]. A further study found no significant effect of IVIG compared with placebo after a 6-week treatment period, although a subsequent 6-week open-LABEL study with IVIG showed positive trends [76]. IVIG is used mainly in myasthenia gravis exacerbation and crisis.

IVIG use in treatment of other conditions

Research into the use of IVIG in other conditions is ongoing [77]. These include relapsing remitting MULTIPLE SCLEROSIS, dermatomyositis, polymyositis, anti-neutrophil cytoplasmic Ab-positive systemic vasculitis refractory to standard immunosuppressive therapy, SYSTEMIC LUPUS ERYTHEMATOSUS, Sjögren's syndrome, anti-phospholipid syndrome, fibrosis-associated disorders, Steven Johnson syndrome and toxic epidermal necrolysis. In addition, IVIG may be of benefit in the treatment of graft-*versus*-host disease and Ab-mediated transplant rejection, post-polio syndrome, narcolepsy and even stroke. The use of IVIG in Alzheimer's disease is also receiving particular attention and is the focus of recent studies [78–80].

Mechanisms of action of IVIG

Historically, the primary use of IVIG was as a reconstitution therapy for people lacking the capacity to produce ANTIBODIES in sufficient amounts to protect themselves from microbial infection. In those patients, protection is mediated by the neutralisation and opsono-phagocytic effects of the four different IgG subtypes described above. If, however, IVIG is used in autoimmune or chronic inflammatory diseases, the beneficial effects are due to the interaction of specific Ab binding to, and thus modulating, humoral and cellular constituents of the dysregulated IMMUNE SYSTEM in the patient.

Effects on soluble factors in the patient's circulation include:

- The direct binding of pathological AUTOANTIBODIES by their anti-IDIOTYPES in IVIG, leading to a reduction in autoantibody-mediated PHAGOCYTOSIS and damage to self structures.
- The binding of potentially detrimental COMPLEMENT COMPONENTS like C3b, C4b, C3a and C5a. This is a mechanism termed complement scavenging. This binding is believed to attenuate complement deposition and the ensuing damage inflicted by complement-mediated CYTOTOXICITY and concurrent INFLAMMATION [16]. Improvement of disease symptoms in patients with dermatomyositis was at least partially attributed to the inhibition of C3 uptake by cells [17].

Mechanisms directed towards immune cells include:

- Competitive FcR blockade on NEUTROPHILS and a shift in expression from activating to inhibitory FcR expression on DENDRITIC CELLS and MONOCYTES/MACROPHAGES.
- Interaction of NAb in IVIG with immunoregulatory molecules, such as B/T CELL RECEPTORS, cytokine RECEPTORS, human leukocyte antigen (HLA) molecules and sialic acid-binding Ig-like LECTINS (Siglecs) [81], thereby reducing proinflammatory cytokine production, the expansion of autoreactive B cells, and the number of potentially harmful effector T cells.
- The modification of dendritic cell function in favour of an anti-inflammatory state, including the production of IL-10, the prevention of dendritic cell-mediated self-reactive T cell activation and general T cell priming.

Indeed, many more MECHANISMS OF ACTION are described in the scientific literature and have recently been reviewed [82–85].

Adverse reactions to IgG therapy

Infusion of IVIG is considered safe and normally well tolerated. Occasionally, however, it can be associated with side effects (adverse drug reactions, ADR), which are most often transient and mild in nature. ADR occur more frequently at the initiation or resumption of therapy and are often related to the infusion rate [86, 87].

Infusion-related systemic adverse reactions

Systemic or generalised ADR include 'flu-like' symptoms such as headache, fatigue, chills, fever, nausea, vomiting, flushing, dizziness, back pain or lower abdominal pain, myalgia, arthralgia and changes in blood pressure. The molecular mechanisms leading to these symptoms are not really clear, but there is evidence to suggest that the reactions could be caused by INFLAMMATORY CYTOKINES such as TNF-α, IL-6 and IL-8, whose production may be induced by the IVIG itself [88] and/or by immune complexes, which may be formed by the infused IgG molecules. Generalised reactions to IVIG vary in severity. Lowering the infusion rate or temporarily interrupting the infusion may be important therapeutic measures for mild-to-moderate ADR.

Pre-medication (e.g. paracetamol, non-steroidal anti-inflammatory drugs or ANTI-HISTAMINE) is often used to prevent generalised reactions.

Experienced clinicians point out that the maximally tolerated infusion rate differs widely between individuals and may depend on the medical history of the patient. In clinical studies, the above described reactions occur in 5–30% of the infusions overall, as stated in the manufacturers' prescribing information. This number contrasts strongly with the results of a study, which followed 'experienced' immunodeficiency patients over 2 years and found an overall ADR rate of 0.8% of the infusions. Only patients who had received at least six IVIG infusions were entered into the study. The same publication clearly confirmed that the occurrence of generalised reactions in immunodeficiency patients is frequently associated with intercurrent infections [89].

Hypersensitivity and anaphylactoid reactions

True allergic/anaphylactic reactions (i.e. IgE-mediated) are not expected with homologous IgG preparations. However, ADR do occur, which are clinically indistinguishable from anaphylactic reactions. They may be called anaphylactoid reactions and they respond to the same therapeutic approach: the infusion is to be stopped and usually, ANTIHISTAMINES and steroids are administered. The need for adrenaline (epinephrine) is very rarely reported.

IgA deficiency is one of the best known causes for such anaphylactoid reactions [90]. About 40% of patients with absolute IgA deficiency have developed Ab against IgA. For these patients, it is advisable to select an IVIG product containing only trace amounts of IgA in an attempt to minimise the risk for such reactions.

For patients who experience moderate to severe systemic reactions after IVIG products, s.c. administration of IgG may be a good alternative [87, 90].

Aseptic meningitis

Headache is the most common reaction to IVIG treatment. Severe headache, in combination with rigidity of the neck, vomiting and photophobia may occur as an infrequent complication of IVIG. This phenomenon, which is called acute aseptic meningitis occurs usually with a latency of several hours to 2 days following IVIG treatment. Analysis of the CSF is negative for pathogens, although invasion of white blood cells, predominantly GRANULOCYTES, is frequently observed in patients suffering from aseptic meningitis. The aetiology of this adverse reaction is unclear. However, patients with a history of migraine seem to be at higher risk for aseptic meningitis [91].

Haemolytic reactions

IVIG products can contain blood group ANTIBODIES, which may act as haemolysins and induce *in vivo* coating of red blood cells (RBC) with IgG. Haemolytic anaemia can develop subsequent to high-dose IVIG therapy in patients with blood group A, B, and AB due to enhanced RBC sequestration. The majority of reports on haemolytic reactions are associated with the more "modern" IVIG products after high cumulative total doses [92, 93].

Thromboembolic events

There is evidence of an association between IVIG administration and rare thromboembolic events such as myocardial infarction, stroke, pulmonary embolism and deep vein thromboses in at-risk patients. While a common hypothesis relates to a relative increase in blood viscosity through the high influx of immunoglobulin, the significant difference between products [94] rather points to another, yet unknown mechanism.

Skin reactions

There are different types of skin reactions, which might be sub-classified into immediate and delayed reactions. Immediate skin reactions (reported as rash or urticaria) are characteristic of acute HYPERSENSITIVITY reactions and are often their only symptom.

Rare, delayed skin reactions present as an eczematous reaction with pruriginous maculopapular lesions involving the whole body. They typically begin at the palms and soles with dyshidrotic lesions about 5–10 days after the start of high-dose IVIG administration. This type of reaction may reoccur at rechallenge, even more intensively [95].

Renal failure

Osmotic nephrosis leading to renal failure may develop after IVIG therapy due to sugar excipients in some IVIG products [96]. Products containing sucrose account for a disproportionate share of the total number of such cases, but products containing maltose or glucose have also been associated with renal failure [97]. Products not containing any sugar excipients do not appear in scientific literature reviews of case reports with renal failure [98, 99].

Viral safety of immunoglobulins

The importance of viral safety has long been realised with all products originating from human plasma. Pathogen safety is of particular importance with IVIG preparations. Patients receiving these products have impaired immune defences and often receive these preparations for many years and sometimes at high or very high doses. The increase in reported cases of acute HEPATITIS C VIRUS in patients receiving IVIG therapy in the early 1980s led to the development of more rigorous screening and manufacturing processes for IVIG production.

Viral safety is generally based on three measures: donor selection, donation testing and manufacturing. Of critical importance is the manufacturing step (see Box 8).

Donor selection

Prospective donors are very carefully screened prior to donation. This includes the completion of detailed questionnaires, a medical examination, and for repeat donors, regular physical examination. In particular, recent concerns regarding the spread of variant Creutzfeldt-Jakob disease or spongiform encephalopathies has meant that the questionnaires include sections aimed at eliminating those donors that may have been exposed to either of these diseases. In the US, even careful selection of the positioning of the plasmapheresis centres has been employed, avoiding areas where there may be a higher prevalence of infected donors [86].

Box 8

The safety strategy for plasma products is based on three measures, namely donor selection, donation and pool screening (serological and nucleic acid testing), and the reduction throughout the manufacturing process of viruses potentially present in the starting material. A manufacturing process encompasses multiple steps that reduce pathogens that are potentially present. These steps complement each other by representing different mechanisms of virus reduction, which should ideally be used in succession. The three mechanisms are partitioning, inactivation and elimination based on size.

Donation testing

Three decades ago, higher rates of markers for transfusion-transmitted infectious diseases were repeatedly found among remunerated donors compared with non-remunerated donors. As a consequence, a safety gap between source and recovered plasma was claimed. This is no longer the case due to the voluntary introduction by the source plasma collecting and manufacturing industry of the International Quality Plasma Program (IQPP) and the Quality Standards of Excellence, Assurance and Leadership (QSEAL).

Donor sera are tested not only with Ab against certain viral pathogens but also by nucleic acid procedures such as polymerase chain reaction; the latter picking up viral genomes before an Ab response has been raised.

Immunoglobulin manufacture

Manufacturers of Ig are required to show that their manufacturing processes inactivate and remove both known blood-borne pathogens as well as novel virus contaminants. By developing scaled-down versions of the production processes, manufacturers can spike samples with viruses and ensure their inactivation and/or removal. Virus removal or inactivation is expressed in the form of logarithmic reduction factors, calculated as the decimal logarithm of the ratio of virus titre spiked to the virus titre recovered after the manufacturing step. Validation of virus partitioning relies on the ability to establish acceptable virus balance or to explain and substantiate the mechanism of virus reduction. For example, in a virus fractionation step, the sum of virus titres recovered from the supernatant and the precipitate should equal the titre of the spiked virus. For virus inactivation processes, the kinetics of decline in infectivity must be shown. Virus reduction steps may include virus elimination, virus inactivation or even a combination of both. Either is acceptable as long as a certified validation procedure takes place.

As highlighted in Box 8, the three mechanisms applicable within a manufacturing process are partitioning, inactivation and virus filtration (see Tab. 4).

Partitioning

Protein precipitation steps followed by filtration or centrifugation are widely used during the purification of plasma proteins and can be very successful at removing the viruses to the waste fractions. This includes the removal of small, non-enveloped viruses that may be resistant to inactivation steps such as pasteurisation or solvent/detergent treatment. It is also one of the key methods for reducing theoretical contamination of IVIG by mis-folded prion proteins (PrP^{Sc}). One commonly used partitioning process is depth filtration in the presence of filter aids, which is usually applied after precipitation of plasma proteins. The use of column chromatography also relies on partitioning as the principal mechanism. When using chromatography, a careful validation of the columns, taking into account the resin's expected life span in production, is vital to ensure no significant loss of partitioning EFFICACY over time.

Table 4. Virus reduction methods used in large-scale manufacturing of plasma products

Mechanism	Principle (examples)	Methods (examples)	Effective against
Partitioning (separation into various physical phases)	Solubility Adsorption Ionic interactions	Cryoprecipitation Cold ethanol fractionation Octanoic acid fractionation Depth filtration Chromatography	Depends on virus characteristics such as charge, hydrophobicity, etc
Inactivation (alteration of structures that are essential for the virus infectivity)	Disintegration of virus (membrane) integrity Alteration of viral structural proteins (DNA, RNA strand breaks)	Solvent/detergent treatment Pasteurisation Dry heat treatment pH 4 incubation Octanoic acid incubation UV- or γ-irradiation	Enveloped viruses Low pH incubation also inactivates some non-enveloped viruses such as B19V and encephalomyocarditis virus Pasteurisation (heat) UV- and γ-irradiation are effective against a variety of non-enveloped viruses
Elimination based on size	Virus filtration (formerly termed nanofiltration)	Tangential or dead-end filtrations	All viruses; depending on virus size and selected membrane pore size

Inactivation

The most commonly employed methods of virus inactivation are low pH, pasteurisation, octanoic acid incubation or solvent/detergent treatment. While octanoic acid incubation and solvent detergent are effective against enveloped viruses only, low pH has been demonstrated to also inactivate B19V and picornavirus encephalomyocarditis virus. Pasteurisation inactivates an even broader range of non-enveloped viruses. Validation of virus inactivation must include data on the inactivation kinetics.

Virus filtration

Previously known as nanofiltration, this involves the removal of viruses based on size; it is effective at removing viruses regardless of any other physicochemical properties. While filters of 35 nm meet current production standards, recent studies have shown that reducing the size of the filters to approximately 20 nm enhances the capacity to remove smaller viruses and even prions [100, 101]. The latter is particularly important, as size exclusion, along with partitioning, form the principal methods of removing PrPSc from plasma products. This is also an important consideration when looking to the future and the removal of yet-unknown viruses.

Summary

Humoral immunity is a vital part of the human immune response. Ig molecules are fundamental to this humoral immune response. The five Ig ISOTYPES and their subclasses have a multitude of effector functions and work very closely together to fight infection. When this humoral immunity is compromised, homologous Ig preparations may be used, and considerable advances in the last century have enabled significant and important improvements in the therapeutic Ig formulations available today. Continual improvements in therapeutic Ig manufacture have led to the development of preparations devoid of aggregates, with Fc effector function, and for which IgG is ensured as the main active component. These developments have also allowed changes in

the route of administration from i.m., which was originally used, to i.v. administration and, more recently, s.c. administration. These changes have significant advantages for patients and there are now at least 11 IVIG and 5 SCIG preparations currently available for therapeutic use. Recent research has also led to the development of ready-to-use Ig solutions stable at room temperature, offering increased convenience for the patient and healthcare provider alike. Over the coming years it is envisaged that additional products will be made available.

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Recommended websites

The National Organization Dedicated to Research, Education and Advocacy for the Primary Immune Deficiency Diseases: Immune Deficiency Foundation: *www.primaryimmune.org* (accessed November 2009)

The Centre for Immunoglobulin Therapy: *www.immunoglobulin-therapy.org/* (accessed November 2009)

World Health Organization: *www.who.int* (accessed November 2009)

European Medicines Agency: *www.emea.eu.int* (accessed November 2009)

US Food and Drug Administration, Centre for Biologics Evaluation and Research: *www.fda.gov/cber* (accessed November 2009)

Paul-Ehrlich Institute: *www.pei.de* (accessed November 2009)

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Anti-allergic drugs

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Introduction

ALLERGY is defined as a disease following a response by the IMMUNE SYSTEM to an otherwise innocuous antigen. Allergic diseases include allergic rhinitis, atopic dermatitis, systemic ANAPHYLAXIS, food ALLERGY, allergic ASTHMA and acute urticaria and are mediated by unwanted type-I HYPERSENSITIVITY reactions (see chapter A9) to extrinsic ALLERGENS like pollen, house dust, animal dander, drugs and insect venom. These diseases are characterised by the production of IgE ANTIBODIES to the allergen that bind to the high-AFFINITY IgE RECEPTOR, FcεRI, on mast cells and BASOPHILS. Binding of allergen to IgE cross-links these RECEPTORS and causes the release of chemical mediators from MAST CELLS, leading to the development of a type-I HYPERSENSITIVITY reaction (Fig. 1). This acute response is often followed by a late and more sustained inflammatory response characterised by the recruitment of other EFFECTOR CELLS such as EOSINOPHILS and T helper type-2 (Th2) LYMPHOCYTES. Among the mainstays in the drug treatment of allergic INFLAMMATION, glucocorticosteroids remain the most potent inhibitors and the reader is referred to chapter C13 for detailed discussion of these drugs. The use of SPECIFIC IMMUNOTHERAPY in severe allergies is considered in chapter C5. This present chapter focuses on anti-allergic drugs that specifically TARGET the activation of the mast cell or block the effects of its chemical mediators, in particular HISTAMINE.

Disodium cromoglycate and nedocromil sodium (cromones)

In the 1960s, Dr Roger E. C. Altounyan (1922–1987), a British physician and pharmaceutical company Fisons, first discovered, in an unusual manner, that disodium cromoglycate (also called cromolyn sodium) possessed an anti-ASTHMA action. He induced an ASTHMA attack in himself by inhaling animal dander ANTIGENS, and showed that cromoglycate afforded protection against this bronchial provocation. Disodium cromoglycate was introduced as an anti-allergic drug in 1968. Many companies tried to find improved versions of this compound, using the chemical structure as a starting point, but most of these attempts failed. Nedocromil was discovered and introduced 20 years later by Eady.

The exact mechanism of action of disodium cromoglycate and the related drug, nedocromil sodium, remains unclear, but their clinical activity probably represents a combination of effects. It was originally suggested that these non-steroidal anti-inflammatory drugs act as mast cell stabilisers [1]. However, although these drugs can prevent HISTAMINE release from MAST CELLS, it has been demonstrated that this effect is not the basis of their action in allergic ASTHMA. Other compounds that inhibit mast cell HISTAMINE release more potently have not proven to be more effective in the treatment of allergic ASTHMA. Sodium cromoglycate and nedocromil sodium also partly inhibit the IgE-mediated release of other mediators from MAST CELLS, such as prostacyclins and LEUKOTRIENES [2]. In addition, they have been described to exhibit suppressive effects on inflammatory cells, such as MACROPHAGES, MONOCYTES, NEUTROPHILS and EOSINOPHILS, but do not have any direct effects on smooth muscle and they do not inhibit the actions of smooth muscle contractile agonists [3, 4]. Sodium cromoglycate and nedocromil sodium inhibit the influx of inflammatory cells and the release of inflammatory mediators following provocation with non-specific agents, such as cold air and air pollutants [5–7]. Furthermore, they have been reported to

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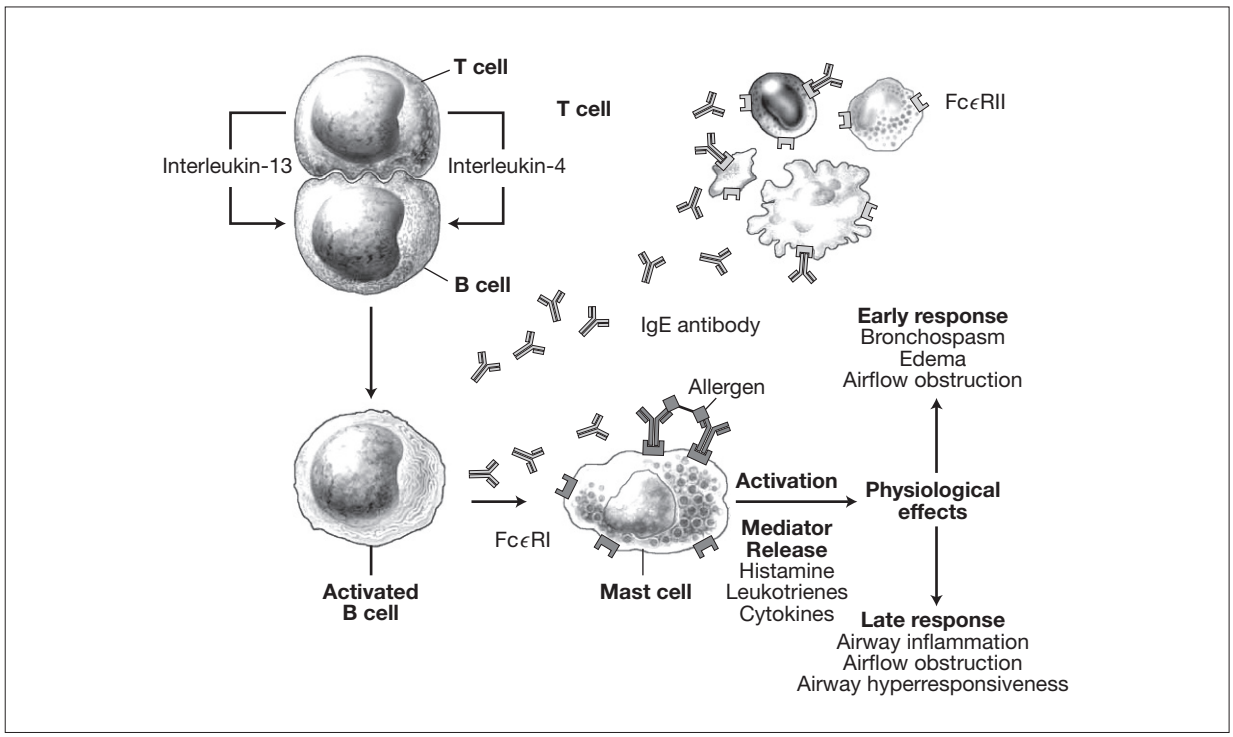

Figure 1. Schematic representation of the induction of IgE synthesis by B lymphocytes
Once formed, IgE antibody circulates in the blood, eventually binding to high-affinity IgE receptors (FcεRI) on mast cells and low-affinity IgE receptors (FcεRII, or CD23) on eosinophils and macrophages. After subsequent encounters with allergen, cross-linking of the high-affinity IgE receptors causes the release of preformed and newly generated mediators. Once present in tissues, mediators may produce various physiological effects, depending on the target organ [52].

depress the exaggerated neuronal reflexes that are triggered by the stimulation of "irritant" RECEPTORS by decreasing neuropeptide release from C fibres and *via* antagonism of tachykinin RECEPTORS [8–10]. A comparison of the activities of sodium cromoglycate and nedocromil sodium on a variety of inflammatory cell types is shown in Table 1. The chemical structures of sodium cromoglycate and nedocromil sodium are depicted in Figure 2.

Mechanisms of action

Many studies have been performed to determine the mechanisms by which cromones inhibit the activation of cells. Since these compounds affect a wide variety of cells it has been assumed that a common mechanism must exist. Experiments have been performed to investigate whether this mechanism is regulated through a specific RECEPTOR or if it is due to the modulation of a second messenger signal.

Initially Ca^{2+} ions were implicated as the TARGET for sodium cromoglycate [11], but this was discounted after it was shown that sodium cromoglycate could also inhibit mast cell activation in the presence of Ca^{2+}-chelating agents [12]. Sodium cromoglycate and nedocromil sodium have been shown to reduce Ca^{2+} influx into cells, although it is believed that they do not interfere directly with Ca^{2+} channels [11]. These compounds have also

Table 1. Comparison of effects of sodium cromoglycate and nedocromil sodium on inflammatory cells

Effect	Nedocromil sodium	Sodium cromoglycate
Mast cells from BAL, lung, conjunctiva, nasal mucosa, gastric mucosa and basophils		
Mediator release following Ascaris Ag or α-IgE Ab inhibited (histamine, PGD_2, LTC_4)	↓	
Release of cytokines (TNF-α)	↓	↓
Release of histamine	↓	↓
Numbers	↓	↓
Macrophages/monocytes		
Release of cytokines (IL-6)	↓	
Release of lysosomal enzymes and oxygen radicals	↓	
Numbers	↓	
Eosinophils		
Numbers in BAL	↓	↓
Number of activated eo´s in submucosa	↓	
Release of mediators (preformed and newly generated)	↓	↓
Chemotactic response to PAF and LTB_4	↓	
Chemotactic response to zymosan-activated serum		↓
Activation	↓	
Survival time in presence of IL-5	↓	
Neutrophils		
Activation	↓	↓
Chemotactic response	↓	↓
Release of mediators (TNF-α, IL-6)	↓	↓
Numbers		↓
Platelets		
Release of cytotoxic mediators	↓	
IgE-mediated activation	↓	
Generation of thromboxane B_2 and IP_3	↓	
Epithelial cells		
Release of 15-HETE	↓	
Release of cytokines (TNF-α, IL-8, GM-CSF) and ICAM-1	↓	
Expression of ICAM-1, VCAM-1, E-selectin	↓	↓
B cells		
IgE Ab formation	↓	↓
T cells		
Numbers	↓	↓
Proliferation (allergen or mitogen induced)	±	±
Endothelial cells		
Expression of ICAM-1, VCAM-1, E-selectin	↓	↓
Sensory nerve (C fibres) activation		
Release of neuropeptides	↓	↓

↓ *reduction;* ± *no change*

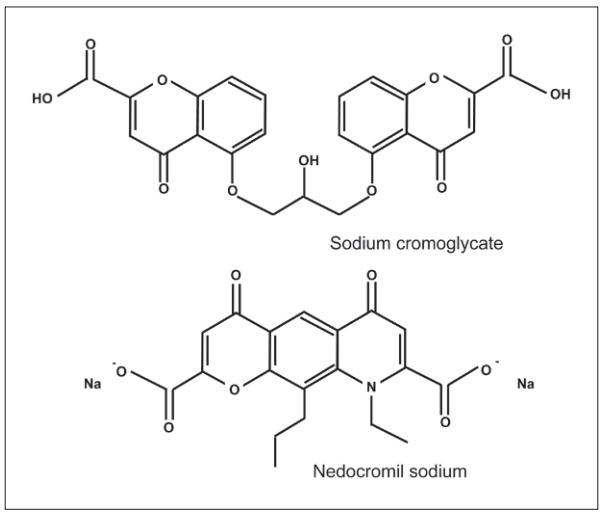

Figure 2. Chemical structures of sodium cromoglycate and nedocromil sodium

been shown to phosphorylate intracellular proteins preceding mediator release from rat peritoneal mast cells; however, this does not hold true for mast cells isolated from the macaque [1]. Additionally, activation of protein kinase C (PKC) has been suggested as the molecular target for sodium cromoglycate [11, 13, 14], although other researchers report an inhibition of PKC activity [15]. The ability of sodium cromoglycate and nedocromil sodium to affect intracellular targets directly is unlikely if we consider the physical properties of these compounds. Both compounds are extremely polar and hydrophilic at pH 7.4, and therefore unlikely to penetrate the cell membrane. Consequently, it was assumed that they must function *via* a cell membrane component, possibly a receptor. Mazurek and colleagues [16–18] have described a "sodium cromoglycate-binding protein" in rat basophil leukaemia (RBL) cells that may be involved in Ca^{2+} mobilisation. Eady and co-workers [19] also identified proteins on rat peritoneal mast cells and Chinese hamster ovary cells that may act as receptors. Until now, the identification of a specific receptor has not yet been established. However, very recently, both cromones were reported to be potent agonists at the G protein-coupled receptor 35 (GPR35), which is up-regulated on mast cells, eosinophils and basophils upon challenge with IgE. GPR35 is a newly discovered receptor on neurons, which inhibits calcium channels and regulates neuronal excitability and synaptic neurotransmitter release, including nociception (pain perception) in dorsal root ganglia in the spinal cord. Agonists at GPR35 include kynurenic acid, a metabolite of tryptophan, and zaprinast, a phosphodiesterase 5 (PDE5) inhibitor. The cromones are both more potent agonists than zaprinast.

There is also an increasing amount of evidence suggesting that modulation of chloride channel activity is possibly a common mechanism to explain the effects of sodium cromoglycate and nedocromil sodium. An accumulation of intracellular Ca^{2+} (Ca^{2+}_i) often precedes cell activation and mediator secretion in many cells. This accumulation of Ca^{2+}_i can result from a Ca^{2+} influx due to a negative membrane potential, which in turn is the result of an inward flow of Cl^- ions through chloride channels. Degranulation is dependent on a sustained elevation of intracellular Ca^{2+}, due to release of Ca^{2+} from intracellular stores and influx of Ca^{2+} ions. A small-conductance chloride channel (0.5–1 pS), identified in rat peritoneal mast cells, can realise this by providing the negative membrane potential necessary for maintaining Ca^{2+} influx and its sustained elevation. The Ca^{2+} current activated by this mechanism is described as I_{CRAC} (Ca^{2+} release-activated Ca^{2+} current). By replacing extracellular Cl^- ions with non-permeant isethionate or gluconate anions, Friis and colleagues [20] were able to inhibit antigen-stimulated histamine secretion from rat peritoneal mast cells, although some histamine secretion still occurred. Sodium cromoglycate can also block intermediate conductance chloride channels on RBL cell membranes [11].

Studies on epithelial cells provide more evidence that sodium cromoglycate and nedocromil sodium affect chloride transport. Alton and colleagues [21] showed that these compounds are able to block the activity of a chloride channel present on the mucosal surface of airway epithelial cells. Moreover, epithelial cells are sensitive to the concentration of solutes in their environment, and chloride currents are believed to be involved in the regulation of cell volume. Nedocromil and cromoglycate can inhibit the chloride current induced in epithelial cells in

response to osmotic changes, thereby inhibiting cell swelling [22]. Furthermore, Paulmichl and colleagues [23] showed that sodium cromoglycate and nedocromil sodium inhibit hypotonic saline-induced activation of a chloride channel in mouse 3T3 fibroblasts. It is now becoming apparent that this chloride channel inhibition by cromones may well be related to their activity as GPR35 agonists, since the chloride channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid is also an agonist of GPR35 in kidney cells [24].

Further, evidence from *in vitro* studies suggests that these compounds affect neuronal chloride transport – chloride efflux from sensory nerves leads to depolarisation and the generation of action potentials. Nedocromil sodium prevents the contraction of guinea pig bronchus that is induced by electric field stimulation in the presence of atropine [10]. Bronchoconstriction is probably mediated by the release of NEUROPEPTIDES from C fibre terminals. This is supported by other studies that show nedocromil inhibition of substance P-induced potentiation of the cholinergic neural responses in rabbit trachea [25] as well as inhibition of tachykinin release [26]. Once again, the presence of GPR35 on neuronal membranes suggests a unifying RECEPTOR-mediated mechanism for cromone action.

Chloride channel activation is a mechanism that occurs when cells are activated. By preventing chloride channel activation, sodium cromoglycate and nedocromil sodium would be expected to maintain cells in a normal resting physiological state, and this is associated with the relative lack of toxicity of these compounds.

Biochemical and pharmacological effects

The anti-inflammatory effects of sodium cromoglycate and nedocromil can result in a number of biological effects:

- Inhibition of mediator release from human MAST CELLS isolated from bronchoalveolar lavage (BAL) fluid and from MAST CELLS derived from lung, conjunctiva and nasal mucosa. Human skin-derived MAST CELLS, however, do not respond to cromoglycate or nedocromil. HISTAMINE secretion by enterochromaffin-like cells from the gastric mucosa can also be inhibited by sodium cromoglycate. Mediator release from EOSINOPHILS, NEUTROPHILS and platelets is also inhibited.
- The numbers of inflammatory cells such as EOSINOPHILS, NEUTROPHILS, BASOPHILS (though not unequivocally confirmed for nedocromil sodium), MAST CELLS, MACROPHAGES and T LYMPHOCYTES are reduced, in both tissues and blood.
- Inflammatory cell infiltration depends on the activating effects of chemotactic factors that are often released by infiltrating inflammatory cells. Cromoglycate and nedocromil can completely suppress the activating effects of chemoattractant peptides on human EOSINOPHILS, NEUTROPHILS and MONOCYTES.
- Inflammatory cell infiltration also depends on the expression of ADHESION MOLECULES. These compounds can inhibit the expression of various ADHESION MOLECULES, such as ICAM-1, VCAM-1 and E-SELECTIN, which are crucial for the passage of inflammatory cells from the blood to peripheral tissues.
- Cell activation and cytokine release from inflammatory cells such as T LYMPHOCYTES, MACROPHAGES and MAST CELLS is inhibited.
- Inhibition of IL-4-induced IgE isotype switching and suppression of IgG4 production, without further effects on B cells that have already undergone switching.
- Sensory nerve (C fibre) activation is inhibited resulting in reduced release of NEUROPEPTIDES such as substance P and tachykinins.
- Survival of platelets can be increased and these compounds inhibit IgE activation of platelets.
- MICROVASCULAR LEAKAGE is reduced, presumably through functional antagonism of tachykinin RECEPTORS.

Pharmacokinetics

Disodium cromoglycate and nedocromil are poorly absorbed from the gastrointestinal tract, and are therefore given locally per inhalation, as either an aerosol (a nebulised solution or in powder form),

or as eye drops. Nedocromil and disodium cromoglygate are not metabolised and are excreted unchanged. Their plasma half-life is approximately 90 minutes.

Clinical indications

Therapeutic studies have revealed and confirmed the clinical EFFICACY, protective effects and high safety/low side-effect profile of these drugs. The diverse clinical effects, including the anti-inflammatory character, of these drugs have been described in detail.

Allergic bronchial asthma

Sodium cromoglycate and nedocromil sodium have been reported to demonstrate protective effects on the immediate ASTHMATIC RESPONSE (IAR) as well as the late ASTHMATIC RESPONSE (LAR) induced by bronchial challenge with allergen. The delayed ASTHMATIC RESPONSE, however, was altered by nedocromil but not by cromoglycate. These compounds not only reduce the numbers of inflammatory cells in the BAL fluid, they also decrease the activation and/or stimulation state of these cells. They also reduce the number of circulating LEUKOCYTES (EOSINOPHILS, NEUTROPHILS and BASOPHILS) during the IAR and LAR following allergen challenge as well as decreasing the activation of circulating T LYMPHOCYTES. Cromoglycate and nedocromil can also inhibit the IAR that occurs during exercise induced ASTHMA and reduce bronchoconstriction due to non-specific hyperreactivity mechanisms. However, not all ASTHMA subjects respond to these drugs and children respond more often than adults [1,27,28].

Allergic rhinitis

Similar to the treatment of allergic ASTHMA, sodium cromoglycate and nedocromil sodium have demonstrated protective effects on the immediate nasal response (INR) as well as the late nasal response (LNR) induced by nasal challenge with allergen. The delayed nasal response, however, was not altered by cromoglycate and only partially prevented by nedocromil. Prophylactic treatment with these compounds significantly reduces inflammatory cell infiltration and epithelial cell numbers as determined by cytological analysis of nasal secretions following allergen challenge in patients with allergic rhinitis [29]. DEGRANULATION of MAST CELLS, BASOPHILS and EOSINOPHILS is inhibited and the expression of ICAM-1 on epithelial cells is down regulated [30,31].

Allergic conjunctivitis

The immediate and late responses to allergen challenge are prevented in the eye. Sodium cromoglycate and nedocromil sodium inhibit the emergence of conjunctival oedema and erythema and reduce mast cell DEGRANULATION as well as vascular leakage [32–34].

Food allergy

Adverse reactions to food, including ALLERGY, can lead to unwanted organ responses. The various organ responses to food ingestion challenge can be inhibited by sodium cromoglycate treatment. This compound significantly inhibits immediate and late types of asthmatic, nasal, paranasal sinus, middle ear, conjunctival, migraine, atopic eczema, urticarial and Quincke's oedema responses to food ingestion ALLERGY [1,29,35].

It can be concluded that sodium cromoglycate and nedocromil sodium are effective drugs in the prophylaxis of allergic bronchial ASTHMA, allergic rhinitis, allergic conjunctivitis and related allergic disorders, with little difference between the two agents [36]. However, some authors suggest that it is not justified to recommend sodium cromoglycate as a first line prophylactic agent in childhood ASTHMA, apart from the treatment of mild symptoms [37,38].

Unwanted effects

Unwanted effects are infrequent and consist predominantly of the effects of irritation in the upper airway. HYPERSENSITIVITY reactions have been reported and include urticaria, bronchospasm, angio-oedema and ANAPHYLAXIS, but these are uncommon.

Histamine receptor antagonists

HISTAMINE was first identified in 1910 by Sir Henry Hallett Dale and the first HISTAMINE RECEPTOR antagonists were synthesised over 20 years later. Early ANTI-HISTAMINE studies were qualitative, for example, by demonstrating their effectiveness in protecting against HISTAMINE-induced bronchospasm. Nevertheless, these studies introduced compounds, such as mepyramine, that remain major ligands to define HISTAMINE RECEPTORS. The first generation ANTI-HISTAMINES were introduced to clinical use in the 1940s. It became apparent in the 1950s, however, that there were multiple HISTAMINE RECEPTORS, and research still continues to identify novel HISTAMINE RECEPTORS. The early ANTI-HISTAMINES were found to be non-selective antagonists of H1 RECEPTORS and all exerted CENTRAL NERVOUS SYSTEM (CNS) side effects, including drowsiness and psychomotor impairment. The development of second and third generation H1 RECEPTOR antagonists resulted in anti-allergic drugs with increased RECEPTOR SPECIFICITY and potency, and reduced CNS penetration. In addition, these newer agents exert broader anti-allergic effects.

Histamine

HISTAMINE [2-(4-imidazolyl)ethylamine or 5-aminoethylimidazole] plays a significant role in the regulation of physiological processes and is an important mediator during allergic reactions. It is synthesised from L-histidine by histidine decarboxylation and stored in various cells, including MAST CELLS, BASOPHILS, neurones and enterochromaffin-like cells. Other cells, predominantly from the haematopoietic lineage, can also synthesise and secrete HISTAMINE, although these cells lack specific storage granules. HISTAMINE can closely mimic the anaphylactic response that usually results from an antigen-ANTIBODY reaction in sensitised tissue. Once released, HISTAMINE can be metabolised by diamine oxidase (DAO) and HISTAMINE *N*-methyltransferase (HMT). The effect of HISTAMINE is produced by its action on specific RECEPTORS, which are subdivided into several groups (H_1, H_2, H_3, and H_4 RECEPTORS). All subtypes are members of the seven membrane-spanning G protein-coupled RECEPTOR (GPCR) family.

Characterisation of histamine receptors

The RECEPTOR subtype determines the biological effects of HISTAMINE. H_1 RECEPTORS are expressed on most smooth muscle cells, endothelial cells, adrenal medulla, heart and CNS. They have also been reported to be expressed on bronchial epithelial cells, fibroblasts, T cells, MONOCYTES, MACROPHAGES, DENDRITIC CELLS and B cells. Their stimulation leads to smooth muscle contraction, stimulation of NO formation, endothelial cell contraction, stimulation of hormone release, negative ionotropism, depolarisation, increased neuronal firing and increased vascular permeability. Stimulation of H_1 RECEPTORS can also lead to pro-inflammatory reactions, such as induction of the expression of ADHESION MOLECULES on endothelial cells, the production of CYTOKINES by these cells and the induction of COSTIMULATORY MOLECULES on DENDRITIC CELLS. H_1 RECEPTOR expression can be modified during inflammatory reactions. H_2 RECEPTORS are expressed on parietal cells in the gut, vascular smooth muscle cells, heart, suppressor T cells, NEUTROPHILS, CNS and BASOPHILS. Their stimulation triggers gastric acid secretion, vascular smooth muscle relaxation, positive chronotropic and ionotropic effects on cardiac muscle, inhibition of lymphocyte function, basophil CHEMOTAXIS and other immune responses. H_3 RECEPTORS are found mainly on cells in the CNS and PERIPHERAL NERVOUS SYSTEM as pre-synaptic RECEPTORS. They have also been identified on endothelium and enterochromaffin cells. These RECEPTORS control release of HISTAMINE and other neurotransmitters, such as ACETYLCHOLINE and dopamine, from neurones. H_4 RECEPTORS, sharing 37% amino acid homology with H_3 RECEPTORS, have been described more recently and are primarily expressed on cells of the haemopoietic lineage and on immunocompetent cells such as MAST CELLS, BASOPHILS, T cells, DENDRITIC CELLS, NEUTROPHILS and EOSINOPHILS, as well as in the spleen and thymus. Stimulation of H_4 RECEPTORS mediates CHEMOTAXIS of MAST CELLS, NEUTROPHILS, EOSINOPHILS and DENDRITIC CELLS and regulates cytokine release from $CD8^+$ T cells [39–45].

H_1 RECEPTOR antagonists are clinically effective when used to treat inflammatory and allergic reactions. The main clinical effect of H_2 RECEPTOR antagonists is on gastric secretion. The clinical relevance of H_3 RECEPTOR antagonists is still being explored, although they appear to be effective in the treatment of CNS disorders. Neither H_2 RECEPTOR antagonists nor H_3 RECEPTOR antagonists are considered to be clinically effective in anti-allergic therapies. H_4 RECEPTOR antagonists, such as JNJ7777120, are being developed and are showing considerable potential for the treatment of allergic diseases such as allergic rhinitis, ASTHMA, as well as for AUTOIMMUNE DISEASES like RHEUMATOID ARTHRITIS and chronic pain in the future [42,45].

Mechanisms of action of H_1 receptor antagonists

The term ANTI-HISTAMINE conventionally refers to H_1 RECEPTOR antagonists and these drugs are discussed in this section. The EFFICACY of these drugs is attributed principally to down-regulation of H_1 RECEPTOR activity. In Table 2, a number of first, second and third generation H1 RECEPTOR antagonists are shown.

Signal transduction by H_1 RECEPTORS (and also for H_4 RECEPTORS) occurs through the hydrolysis of phosphatidylinositols. HISTAMINE binds to the RECEPTOR, which in turn activates the $G_{\alpha q}$ protein ($G_{i/o}$ protein of H_4 RECEPTORS). Activation of these G proteins precedes activation of phospholipase C (PLC), which cleaves phosphatidylinositol bisphosphate (PIP_2) to form inositol tri-phosphate (IP_3) and diacylglycerol (DAG). IP_3 activates IP_3 RECEPTORS on the endoplasmic reticulum, causing the release of Ca^{2+}_i, as depicted in Figure 3. The various biological effects follow the rise in Ca^{2+}.

Pharmacological effects of H_1 receptor antagonists

H_1 RECEPTORS modulate inflammatory and allergic responses by controlling NO formation and smooth muscle and endothelial cell contraction, which subsequently result in increased vascular permeability. HISTAMINE can also stimulate sensory nerve endings, thereby causing itching of the mucosa and skin through stimulation of C fibres. Regulation of the transcription factor NF-κB and subsequent generation of ADHESION MOLECULES (ICAM-1 and P-selectin) and CYTOKINES (IL-6, IL-8, GM-CSF, RANTES) are also H_1 RECEPTOR dependent. The pharmacological actions of H_1 RECEPTOR antagonists are therefore useful for the inhibition of contraction of the smooth muscle and the inhibition of HISTAMINE-induced vascular permeability. Additionally, H_1 RECEPTOR antagonists inhibit the constitutive activation of NF-κB, which results in an inhibition of cytokine production. H_1 RECEPTOR antagonists inhibit HISTAMINE-induced bronchospasm in the guinea pig, but they are not effective in decreasing allergen-induced bronchospasm in human airways. Furthermore, first generation H_1 RECEPTOR antagonists can exhibit anti-serotonergic, anti-emetic and/or anti-cholinergic characteristics, depending on the particular H_1 RECEPTOR antagonist used. Second generation H_1 RECEPTOR antagonists, however, are more selective, have minimal sedative effects and have little AFFINITY for muscarinic cholinergic, α-adrenergic or serotoninergic RECEPTORS, although they still have dose-related adverse effects at high doses. Third generation H_1 RECEPTOR antagonists are either active metabolites or enantiomers of second generation compounds and show reduced adverse effects.

Suppression of mediator release from MAST CELLS and BASOPHILS by second and third generation H_1 RECEPTOR antagonists are thought to occur independently of the H_1 RECEPTOR. Neither MAST CELLS nor BASOPHILS express H_1 RECEPTORS on their surface. However, MAST CELLS, BASOPHILS and EOSINOPHILS all express the H_4 RECEPTOR on their surface. Recently, a variety of known H_1 RECEPTOR antagonists were shown to be weaker partial agonists at H_4 RECEPTORS, which would help to explain their broader spectrum of activity [46].

Pharmacokinetics

Most H_1 RECEPTOR antagonists are given orally, some are given as nose sprays or eye drops, they are well absorbed and reach their peak effect in 1–2 hours. The duration of activity depends largely on whether

Table 2. First, second and third generation H_1 receptor antagonists

Antagonist	Disorder
First generation:	
Clemastine	Anaphylactic reactions to insect bites or food allergy
Dexchlorofeniramine	Allergic conditions
Dimethindene	Allergic conditions
Diphenhydramine	Rhinitis / urticaria
Emedastine	Conjunctivitis / rhinitis
Hydroxyzine	Pruritis / chronic urticaria
Mebhydroline	Allergic conditions
Oxatomide	Allergic conditions
Promethazine	Allergic conditions / anaphylactic shock
Second generation:	
Acrivastine	Allergic rhinitis/ hay fever
Astemizole	Urticaria
Cetirizine	Allergic rhinitis / conjunctivitis/ urticaria
Fexofenadine	Allergic rhinitis / chronic urticaria
Ketotifen	Allergic rhinitis / allergic skin conditions / prophylactic for asthma
Levocabastine	Allergic rhinitis / conjunctivitis
Levocetirizine	Allergic rhinitis / chronic urticaria
Terfenadine	Allergic rhinitis / conjunctivitis / allergic skin disorders
Third generation:	
Azelastine	Allergic rhinitis / conjunctivitis
Desloratidine	Allergic rhinitis
Ebastine	Allergic rhinitis / conjunctivitis
Loratadine	Allergic rhinitis / conjunctivitis / chronic urticaria / pruritis
Mizolastine	Allergic rhinitis / conjunctivitis / urticaria

first, second or third generation drugs are administered, and can vary between 2 hours and a few days. Most of these drugs are widely distributed throughout the body, but the third generation drugs do not pass the blood-brain-barrier. They are largely metabolised in the liver and excreted in the urine.

Clinical indications

H_1 RECEPTOR antagonists can effectively control allergic disorders with mild symptoms, especially of the upper airways and skin. Allergic disorders with more severe symptoms and a complicated clinical picture, such as severe ASTHMA, require other therapies but H_1 RECEPTOR antagonists may be supplemental.

Allergic rhinitis

H_1 RECEPTOR antagonists, administered either orally or topically to mucosal surfaces, are the most frequently used first-line medication for intermittent (seasonal) and persistent (perennial) allergic rhinitis. Non-sedating second-generation H_1 RECEPTOR antagonists such as cetirizine, fexofenadine and loratadine have been proven effective in short- and long-term studies. Also desloratadine, levocetirizine and tecastemizole have been found to be effective in allergic rhinitis. H_1 RECEPTOR antagonists reduce sneezing and rhinorrhoea as well as itchy, watery, red eyes. They reduce itchy nose, palate, or throat, and sometimes reduce nasal congestion. There are very few clinical differences between the H_1 RECEPTOR antagonists. Some

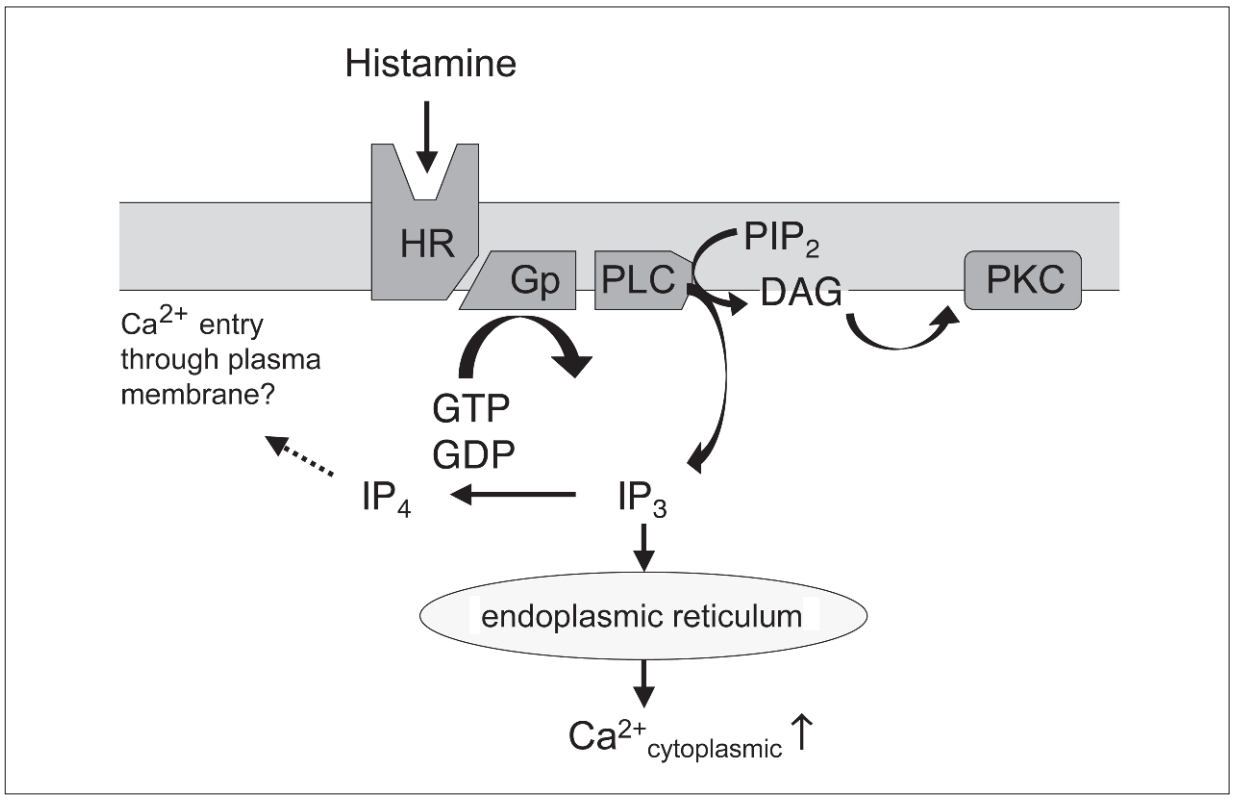

Figure 3. Signalling pathway of H_1 receptor (and possibly H_4 receptor)
Histamine binds to the receptor, which in turn activates the $G_{\alpha q}$ protein ($G_{i/o}$ protein of H_4 receptors). Activation of these G proteins precedes activation of PLC, which hydrolyses IP_3. IP_3 activates IP_3 receptors on the endoplasmic reticulum, causing the release of intracellular Ca^{2+}. The rise in Ca^{2+} is followed by the biological effect. HR, histamine receptor; Gp, G protein; PLC, phospholipase C; PIP_2 phosphatidylinositol bisphosphate; DAG, diacyglycerol; PKC, protein kinase C; GTP, guanosine triphosphate; GDP, guanosine diphosphate; IP_3, inositol triphosphate; Ca^{2+}, calcium iona.

investigators have reported that H_1 receptor antagonists reduce the influx of inflammatory cells into nasal secretion, whereas other investigators report a lack of inhibitory effects.

Mild atopic asthma

Cetirizine, desloratidine and loratidine have been reported to improve mild "seasonal" asthma symptoms, reduce the amount of β_2-agonist usage, and improve pulmonary function. These compounds prevent and relieve allergic inflammation in both the upper and lower airways. H_1 receptor antagonists have not been reported to be clinically effective in the treatment of bronchial asthma and are therefore not a first choice for the treatment of this disorder. They do not affect histamine- or methacholine-induced bronchospasm but they may be useful as additional or supplemental therapy.

Allergic conjunctivitis

This disorder usually occurs as part of an allergic syndrome, i.e. together with allergic rhinitis. If acute symptoms arise the eyes can be treated locally with azelastine, emadine, ketotifen, levocabastine or olo-

patadine. The main symptoms are red, itchy, watery eyes.

Acute and chronic urticaria

This disorder can be treated with oral, H_1 RECEPTOR antagonists. These compounds reduce itching as well as the number, size and duration of urticarial lesions. Erythema may not be completely inhibited because the vascular effects of HISTAMINE are also mediated *via* H_2 RECEPTORS as well as by other vasoactive substances such as proteases, EICOSANOIDS (LEUKOTRIENES, PROSTAGLANDIN E_1) and NEUROPEPTIDES (substance P). First and second generation H_1 RECEPTOR antagonists have been shown to be equally effective. Urticarial vasculitis cannot be satisfactorily treated with H_1 RECEPTOR antagonists.

Treatment of anaphylactic shock

The initial treatment of choice is adrenaline (epinephrine), and treatment of anaphylactic SHOCK can be accomplished with intramuscular or subcutaneous injections of adrenaline. The H_1 RECEPTOR antagonist clemastine may be given intravenously (2 mg) as an adjuvant. H_1 RECEPTOR antagonists may also be useful in the ancillary treatment of pruritis, urticaria and angio-oedema.

Atopic dermatitis (or eczema)

This is often treated with oral H_1 RECEPTOR antagonists that also exhibit a sedative action, in conjunction with TOPICAL GLUCOCORTICOIDS to relieve itching. Second generation H_1 RECEPTOR antagonists are generally less effective than first generation drugs.

Unwanted effects

Most H_1 RECEPTOR antagonists have few unwanted effects when used at the recommended doses, although adverse CNS effects have been observed. The first generation H_1 RECEPTOR antagonists have marked sedative effects due to the fact that they can cross the blood-brain barrier. The second generation H_1 RECEPTOR antagonists are more specific for the H_1 RECEPTOR than first generation drugs, and therefore have little AFFINITY for muscarinic cholinergic, α-adrenergic or serotoninergic RECEPTORS; these compounds have less sedative effects. Third generation H_1 RECEPTOR antagonists have been developed more recently to further reduce adverse effects. Dry mouth, urinary dysfunction, constipation, tachycardia and other unwanted effects are consequently not often observed. Allergic dermatitis following TOPICAL application has been reported.

H_4 receptor antagonists

Increased numbers of MAST CELLS are found in the airways of allergic ASTHMA and allergic rhinitis patients. Hofstra and colleagues [40] showed that stimulation of H_4 RECEPTORS with HISTAMINE mediates cell signalling and mast cell CHEMOTAXIS in a dose-dependent manner. Redistribution of MAST CELLS during allergic episodes may be mediated by this mechanism, indicating that specific H_4 RECEPTOR antagonists may prove useful in the treatment of allergic diseases in the future. Since EOSINOPHILS have also been demonstrated to express H_4 RECEPTORS, it is likely that specific H_4 RECEPTOR antagonists will also inhibit their migration and infiltration of tissues during allergic reactions. Furthermore, H_4 RECEPTORS appear to play a role in the control of cytokine release from $CD8^+$ T cells, monocyte-derived DENDRITIC CELLS, and possibly other cells that express the H_4 RECEPTOR, during inflammatory disorders such as ASTHMA [39, 45].

Some of the currently available H_3 RECEPTOR agonists and antagonists are also recognised by the H_4 RECEPTOR, although they are much less potent for the H_4 RECEPTORS. Using cells transfected with the H_4 RECEPTOR, it has been shown that specific H_1 and H_2 RECEPTOR agonists and antagonists do not bind to the H_4 RECEPTOR. Specific antagonists for the H_4 RECEPTOR are already under development. The non-imidazole, neutral antagonist, JNJ7777120, has a 1000-fold selectivity for H_4 RECEPTORS in comparison to other HISTAMINE RECEPTOR subtypes and inhibits experimental allergic INFLAMMATION in animals. However, it has a short half-life ($t_{1/2}$ = 0.8 h) after oral administration [42, 45]. Several other novel compounds, including

UR-63325, are in early clinical trials for allergic respiratory INFLAMMATION.

Anti-leukotrienes

In addition to HISTAMINE, IgE-stimulated MAST CELLS release a large number of different inflammatory mediators, including proteases, CHEMOKINES, CYTOKINES and EICOSANOIDS (Fig. 1). Currently, apart from the ANTI-HISTAMINES, the anti-LEUKOTRIENES are the only other class of mast cell mediator inhibitors that are in clinical use. As presented in chapter A7, the LEUKOTRIENES (LTs) exert a number of effects that are relevant for allergic respiratory INFLAMMATION, including bronchoconstriction and vasodilation by the cysteinyl-LTs and CHEMOTAXIS and activation of POLYMORPHONUCLEAR LEUKOCYTES by LTB_4. Inhibition of these effects by anti-LT agents, including the 5-LIPOXYGENASE (5-LO) inhibitor, zileuton, and the cysteinyl-LEUKOTRIENE (cys-LT_1) RECEPTOR antagonists, montelukast, zafirlukast and pranlukast, exerts beneficial effects on allergic INFLAMMATION, including ASTHMA, as discussed in greater detail in chapter C5.

In addition to its use in ASTHMA, oral montelukast exhibits comparable EFFICACY and add-on effects to H_1 RECEPTOR antagonists in daytime nasal congestion due to allergic rhinitis, but is less effective than intranasal glucocorticosteroids. Cys-LT_1 RECEPTOR antagonists are ineffective for treatment of allergic inflammatory symptoms outside the airways (conjunctivitis, oral symptoms, eczema and/or urticaria). Montelukast is well tolerated, although rare cases of neuropsychiatric events (e.g. agitation, aggression, anxiousness, dream abnormalities) have been reported [47].

Anti-IgE

Ever since the discovery of the function of IgE more than three decades ago [48], research has been focussed on the selective inhibition of either the activity or the production of IgE. Omalizumab, marketed as Xolair, is a monoclonal ANTIBODY that targets IgE. Xolair is a RECOMBINANT DNA-derived humanised IgG1κ monoclonal ANTIBODY that selectively binds to human IgE. It is a humanised mouse ANTIBODY that contains only 5% amino acid sequence derived from the mouse. The ANTIBODY has a molecular mass of ~149 kDa and is produced by Chinese hamster ovary cells. Xolair has been approved by the FDA for the treatment of adults and adolescents (12 years of age and older) with moderate to severe persistent ASTHMA who have a positive skin test or *in vitro* reactivity to a perennial aeroallergen and whose symptoms are inadequately controlled with inhaled corticosteroids. Xolair is given by subcutaneous injection at a dose of 150–375 mg every 2–4 weeks based upon pre-treatment serum IgE levels and total body weight.

Biochemical and pharmacological effects

Xolair binds to free IgE at the FcεRI binding site on the Cε3 domain of the IgE ANTIBODY, thereby inhibiting the binding to FcεRI on the surface of mast cells, BASOPHILS and other FcεRI$^+$ cells (Fig. 4). The reduction of surface-bound IgE on FcεRI-bearing cells prevents or limits the release of chemical mediators of the allergic response. Levels of serum free IgE decrease by >90% from pre-treatment values within 24 hours after subcutaneous administration of Xolair. In addition, the expression of FcεRI appears to be down-regulated as demonstrated on BASOPHILS after continued (3 month) Xolair treatment [49].

Clinical trials

Several clinical studies using RECOMBINANT humanised monoclonal ANTIBODY to human IgE (E25, Xolair, Omalizumab) have been conducted and published (reviewed in [50]).

The approval of Xolair was based upon EFFICACY data from several multicentre placebo-controlled phase III clinical trials with symptomatic patients with moderate to severe persistent ASTHMA who had a positive skin test reaction to a perennial aeroallergen [51–53]. All patients were also being treated with inhaled corticosteroids and short-acting β-agonists.

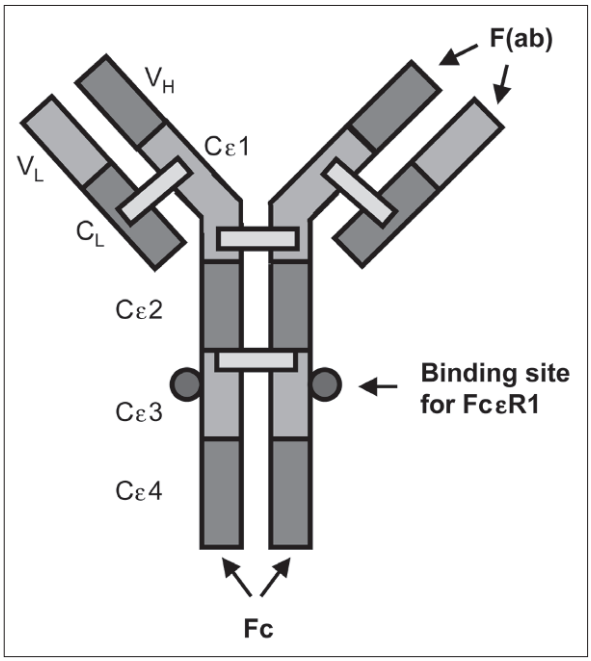

Figure 4. Structure of IgE and position of binding site for FcεRI [50]

Efficacy in these trials was based upon the number of asthma exacerbations per patient, defined as a worsening of asthma that required treatment with systemic corticosteroids or a doubling of the baseline dose of inhaled corticosteroid. Study results showed that the number of exacerbations per patient was reduced in patients receiving Xolair compared with placebo. Reduction of asthma exacerbations was not observed in Xolair-treated patients who had baseline FEV_1 >80% or in patients who required oral steroids as maintenance therapy. Comparative clinical studies between Xolair and other agents used to treat asthma are currently not available. Xolair has not been shown to alleviate asthma exacerbations acutely and it is not indicated for the treatment of bronchospasm or status asthmaticus.

The following effects of Xolair have been observed in clinical trials with allergic asthma patients:

- >90% drop in serum IgE levels within hours after injection [54,55].
- Partial reduction of drop in FEV_1 during early- and late-phase asthmatic response after allergen challenge [56,57].
- Reduction of asthma symptom scores and increase of quality-of-life scores; decreased use of β-agonists after high dose of Xolair; reduction of oral or inhaled steroid use [52,54].
- Reduction of asthma exacerbations during stable- and steroid-reduction phase; steroid-sparing effect [52,53].
- Steroid-sparing effect in asthmatic children; no change in asthma symptom scores [58].

The following effects of Xolair have been observed in clinical trials with seasonal allergic rhinitis patients:

- No significant difference in daily symptoms scores; no significant difference in the use of rescue medication [55].
- Reduction of rescue anti-histamines; decreased daily nasal symptom severity scores [59].
- Dose-dependent reduction of nasal and ocular symptom severity and duration scores; reduction of rescue anti-histamines [60].

Omalizumab is also under investigation for other IgE-dependent allergic indications, with some suggestion that it may be useful in severe, treatment-resistant atopic dermatitis, chronic urticaria, allergic bronchopulmonary aspergillosis, peanut allergy and prophylaxis for severe insect sting allergies. Larger, randomized studies in well-characterized patients are needed to determine the efficacy of the drug in these conditions.

Unwanted effects

Single- and multiple-dose trials in adults with and without allergic diseases have demonstrated that Xolair is well tolerated. Immune complex formation of Xolair with IgE leads to relatively small complexes (<1000 kDa) that are not complement fixing and do not accumulate within any organ system [61]. The immune complexes have a serum half-life of approximately 20 days and are cleared *via* Fcγ receptors of the reticuloendothelial system [61].

The most commonly reported adverse effect is an urticarial rash, with an incidence of 0.5–0.7%. The rash develops within 1 hour of receipt of the first dose and responds to ANTI-HISTAMINE therapy. Other adverse effects associated with Xolair are headache, fatigue and vertigo. There are no reports of systemic anaphylactic reactions, and no ANTIBODIES to the anti-IgE ANTIBODY have been detected.

Conclusions

The IgE-mast cell pathway plays a central role in the pathogenesis of allergic diseases. It is therefore not surprising that many anti-allergic drugs are targeted at this pathway. These drugs either prevent or reverse allergen-induced mast cell activation by blocking IgE (Xolair) or stabilizing mast cells (cromones) or block the biological effects of the HISTAMINE released upon allergen-induced mast cell activation (ANTI-HISTAMINES). ANTI-HISTAMINES have proven to be safe and successful, although they may not block all effects of mast cells. Cromones can be used as prophylactic drugs for allergic diseases but their clinical EFFICACY is not undisputed. Xolair is very effective in reducing serum IgE levels and the consequent mast cell activation. However, this protein drug needs to be administered parenterally and the cost of treatment is very high compared to other anti-allergic treatments. Thus, Xolair is not the first line of prophylactic treatment for allergic diseases. Anti-LTs have limited therapeutic application beyond their use in ASTHMA.

Summary

ALLERGY is defined as a disease that develops following a response by the IMMUNE SYSTEM to an otherwise innocuous agent. This chapter describes the actions of anti-allergic drugs and therapeutics on diseases such as allergic rhinitis, atopic dermatitis, allergic conjunctivitis systemic ANAPHYLAXIS, food ALLERGY, allergic ASTHMA and acute urticaria. The putative MECHANISMS OF ACTION of disodium cromoglycate and nedocromil sodium (cromones) are discussed in detail as well as the biochemical and pharmacological effects, clinical applications and unwanted effects of these drugs. The characterisation of HISTAMINE RECEPTORS is described and the MECHANISMS OF ACTION as well as the observed biological effects of H_1 and the relatively new H_4 RECEPTORS are explained. Further, the biochemical and pharmacological effects of anti-IgE therapy are described and recent clinical trials with these drugs are briefly reviewed.

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Drugs for the treatment of airway disease

Peter J. Barnes

Introduction

Both ASTHMA and chronic obstructive pulmonary disease (COPD) are characterized by airflow obstruction and chronic INFLAMMATION of the airways, but there are important differences in inflammatory mechanisms and response to therapy between these diseases [1, 2]. This chapter discusses the pharmacology of the drugs used in the treatment of obstructive airways diseases. These drugs include bronchodilators, which act mainly by reversing airway smooth muscle contraction and anti-inflammatory drugs, which in ASTHMA suppress the inflammatory response in the airways. The most effective anti-inflammatory treatment for ASTHMA is corticosteroids, which are covered in chapter C13. In COPD, no effective anti-inflammatory drugs are available, but several new classes of drug are now in development.

Bronchodilators

Bronchodilator drugs have an "anti-bronchoconstrictor" effect, which may be demonstrated directly *in vitro* by a relaxant effect on precontracted airways. Bronchodilators cause immediate reversal of airway obstruction in ASTHMA *in vivo*, and this is believed to be due to an effect on airway smooth muscle, although additional pharmacological effects on other airway cells (such as reduced MICROVASCULAR LEAKAGE and reduced release of bronchoconstrictor mediators from inflammatory cells) may contribute to the reduction in airway narrowing.

Three main classes of bronchodilator are in current clinical use:

- β-Adrenergic agonists (sympathomimetics)
- Theophylline (a methylxanthine)
- Anticholinergics (muscarinic RECEPTOR antagonists)

Drugs such as sodium cromoglycate, which prevent bronchoconstriction, have no direct bronchodilator action and are ineffective once bronchoconstriction has occurred. Anti-LEUKOTRIENES (LEUKOTRIENE RECEPTOR antagonists and 5'-LIPOXYGENASE inhibitors) have a small bronchodilator effect in some patients and appear to act more to prevent bronchoconstriction.

β_2-Adrenergic agonists

Chemistry

The development of β_2-agonists was a logical development of substitutions in the catecholamine structure of noradrenaline and adrenaline (epinephrine). The catechol ring consists of hydroxyl groups in the 3 and 4 positions of the benzene ring (Fig. 1). Noradrenaline differs from adrenaline only in the terminal amine group, which therefore indicates that modification at this site confers β-RECEPTOR selectivity. Further substitution of the terminal amine resulted in β_2-RECEPTOR selectivity, as in salbutamol and terbutaline. CATECHOLAMINES are rapidly metabolized by the enzyme catechol-*o*-methyl transferase (COMT), which methylates in the 3-hydroxyl position, and accounts for the short duration of action of CATECHOLAMINES. Modification of the catechol ring, as in salbutamol and terbutaline, prevents this degradation and therefore prolongs their effect. CATECHOLAMINES are also broken down

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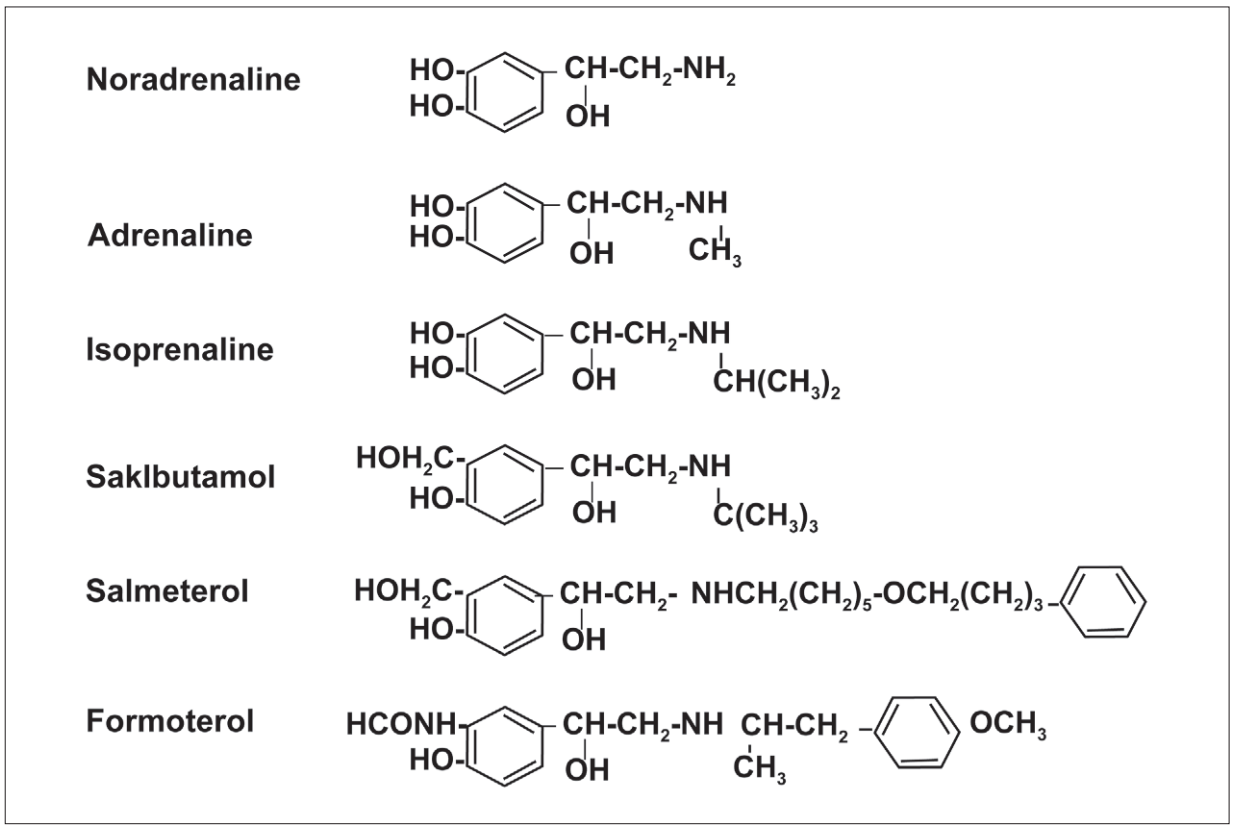

Figure 1. Chemical structure of some adrenergic agonists showing development from catecholamines

in sympathetic nerve terminals and in the gastrointestinal tract by monoamine oxidase (MAO), which cleaves the side chain. Isoprenaline, which is a SUBSTRATE for MAO, is therefore metabolized in the gut, making absorption variable. Substitution in the amine group confers resistance to MAO and ensures reliable absorption. Many other β_2-selective agonists have now been introduced and, while there may be differences in potency, there are no clinically significant differences in selectivity. Inhaled β_2-selective drugs in current clinical use (apart from rimiterol, which is broken down by COMT) have a similar duration of action of 3–6 hours. The long-acting inhaled β_2-agonists salmeterol and formoterol have a much longer duration of effect, providing bronchodilatation and bronchoprotection for over 12 hours [3]. Formoterol has a bulky substitution in the aliphatic chain and has a moderate lipophilicity, which keeps the drug within the membrane close to the RECEPTOR, so it behaves as a slow-release drug. Salmeterol has a long aliphatic chain and its long duration may be due to binding within the RECEPTOR binding cleft that anchors the drug in the binding cleft. Once-daily β_2-agonists, such as indacaterol and vilanterol, with a duration of action >24 h, are now in development [4].

Mode of action

Occupation of β_2-RECEPTORS by agonists results in the activation of adenylyl cyclase *via* the stimulatory G protein (G_s). This increases intracellular cyclic adenosine 3',5'-monophosphate (cAMP), leading to

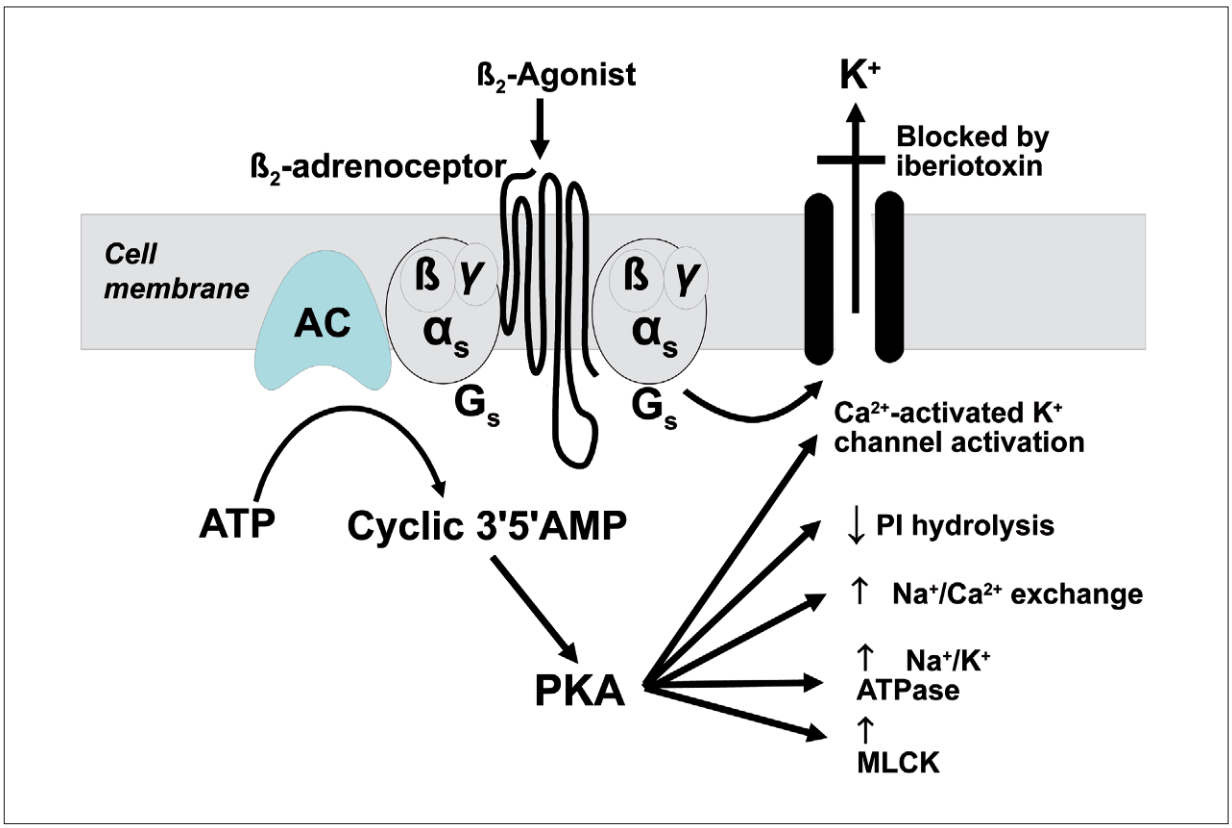

Figure 2. Molecular mechanism of action of β_2-agonists on airway smooth muscle cells
Activation of β_2-receptors (β_2AR) results in activation of adenylyl cyclase (AC) via a stimulatory G protein (G_s) and increase in cyclic 3'5' adenosine monophosphate (AMP). This activates protein kinase A, which then phosphorylates several target proteins, resulting in opening of calcium-activated potassium channels (K_{Ca}) or maxi-K channels, decreased phosphoinositide (PI) hydrolysis, increased sodium/calcium ion (Na^+/Ca^{2+}) exchange, increased Na^+/K^+ ATPase and decreased myosin light chain kinase (MLCK) activity. In addition, β_2-receptors may be coupled directly via G_s to K_{Ca}. ATP: adenosine triphosphate.

activation of a specific kinase (protein kinase A), which phosphorylates several TARGET proteins within the cell, leading to relaxation (Fig. 2). β-Agonists produce bronchodilatation by directly stimulating β_2-RECEPTORS in airway smooth muscle, and *in vitro* relax human bronchi and lung strips (indicating an effect on peripheral airways); *in vivo* there is a rapid decrease in airway resistance. β-RECEPTORS have been localized to airway smooth muscle of all airways by direct RECEPTOR binding techniques and autoradiographic mapping studies.

The molecular mechanisms by which β-agonists induce relaxation of airway smooth muscle include:

- Lowering of intracellular calcium ion (Ca^{2+}) concentration by active removal of Ca^{2+} from the cell and into intracellular stores
- An inhibitory effect on phosphoinositide hydrolysis, which leads to intracellular Ca^{2+} release
- Inhibition of myosin light chain kinase
- Activation of myosin light chain phosphatase

- Opening of a large conductance calcium-activated potassium channel (K_{Ca}), which repolarizes the smooth muscle cell and may stimulate the sequestration of Ca^{2+} into intracellular stores. β_2-RECEPTORS are also directly coupled to K_{Ca} *via* G_s so that relaxation of airway smooth muscle may occur independently of an increase in cAMP.

Recently it has been recognized that several actions of β_2-agonists are not mediated *via* PKA and that there are other cAMP-regulated proteins, such as exchange protein activated by cAMP (EPAC) [5].

β_2-Agonists act as functional antagonists, and reverse bronchoconstriction irrespective of the contractile agent. This is an important property for the treatment of ASTHMA, as many bronchoconstrictor mechanisms (neurotransmitters and mediators) are likely to be contributory. In COPD the major mechanism of action is likely to be reduction of cholinergic neural bronchoconstriction.

β_2-Agonists may have additional effects on airways, and β-RECEPTORS are localized to several different airway cells (Tab. 1). β_2-Agonists may therefore cause bronchodilatation *in vivo* not only *via* a direct action on airways smooth muscle, but also indirectly by inhibiting the release of bronchoconstrictor mediators from inflammatory cells and of bronchoconstrictor neurotransmitters from airways nerves. These mechanisms include:

TABLE 1. EFFECTS OF β-ADRENERGIC AGONISTS ON AIRWAYS

Relaxation of airway smooth muscle (proximal and distal airways)
Inhibition of mast cell mediator release
Inhibition of plasma exudation and airway edema
Increased mucociliary clearance
Increased mucus secretion
Decreased cholinergic neurotransmission
Decreased cough
No effect on chronic inflammation

- Prevention of mediator release from isolated human lung MAST CELLS (*via* β_2-RECEPTORS).
- Prevention of MICROVASCULAR LEAKAGE and thus the development of bronchial mucosal edema after exposure to mediators, such as HISTAMINE and LEUKOTRIENE D_4.
- Increase in mucus secretion from submucosal glands and ion transport across airway epithelium; these effects may enhance mucociliary CLEARANCE, and therefore reverse the defective CLEARANCE found in ASTHMA.
- Reduction in neurotransmission in human airway cholinergic nerves by an action at pre-junctional β_2-RECEPTORS to inhibit ACETYLCHOLINE release. This may contribute to their bronchodilator effect by reducing cholinergic reflex bronchoconstriction.

Although these additional effects of β_2-agonists may be relevant to the prophylactic use of these drugs against various challenges, their rapid bronchodilator action is probably attributable to a direct effect on airway smooth muscle.

Anti-inflammatory effects

Whether β_2-agonists have anti-inflammatory effects in ASTHMA is controversial. The inhibitory effects of β_2-agonists on mast cell mediator release and MICROVASCULAR LEAKAGE are clearly anti-inflammatory, suggesting that β_2-agonists may modify acute INFLAMMATION. However, β_2-agonists do not appear to have a significant inhibitory effect on the chronic INFLAMMATION of asthmatic airways, which is suppressed by corticosteroids. This has now been confirmed by several biopsy and bronchoalveolar lavage studies in patients with ASTHMA who are taking regular β_2-agonists (including long-acting inhaled β_2-agonists). These studies demonstrated no significant reduction in the number or activation in inflammatory cells in the airways, in contrast to resolution of the INFLAMMATION that occurs with inhaled corticosteroids. This is likely to be related to the fact that β_2-agonists effects on MACROPHAGES, EOSINOPHILS and LYMPHOCYTES are rapidly desensitized due to a low density of β_2-RECEPTORS on these cells.

Clinical use

Short-acting β_2-agonists

Inhaled β_2-agonists are the most widely used and effective bronchodilators in the treatment of ASTHMA due to their functional antagonism. When inhaled from pressurized metered dose inhaler (pMDI) or dry powder inhaler (DPI), they are convenient, easy to use, rapid in onset and without significant side effects. In addition to an acute bronchodilator effect, they are effective in protecting against various challenges, such as exercise, cold air and allergen. They are the bronchodilators of choice in treating acute severe ASTHMA, and the nebulized route of administration is as effective as, and easier and safer than, intravenous administration. The inhaled route of administration is preferable to the oral route because side effects are fewer, and it may be more effective. Short-acting inhaled β_2-agonists, such as salbutamol and terbutaline, should be used "as required" by symptoms and not on a regular basis in the treatment of mild ASTHMA, as increased usage serves is an indicator for the need for more anti-inflammatory therapy.

Oral β_2-agonists are occasionally indicated as an additional bronchodilator. Slow-release preparations (such as slow-release salbutamol and bambuterol) may be indicated in nocturnal ASTHMA, but are less useful than inhaled β-agonists because of an increased risk of side effects. The once-daily β_2-agonist bambuterol (a prodrug that slowly releases terbutaline) is effective as an add-on therapy, although systemic side effects may be a problem.

Several short-acting β_2-agonists are available. With the exception of rimiterol (which retains the catechol ring structure and is therefore susceptible to rapid enzymatic degradation), they have a longer duration of action because they are resistant to uptake and enzymatic degradation by COMT and MAO. There is little to choose between the various short-acting β_2-agonists currently available; all are usable by inhalation and orally, have a similar duration of action (usually 3–4 hours but less in severe ASTHMA) and similar side effects. Differences in β_2-selectivity have been claimed but are not clinically important. Drugs in clinical use include salbutamol, terbutaline, fenoterol, tulobuterol, rimiterol and pirbuterol.

Long-acting inhaled β_2-agonists

The long-acting inhaled β_2-agonists (LABA) salmeterol and formoterol have proved to be a major advance in ASTHMA and COPD therapy. Both drugs have a bronchodilator action of >12 hours and also protect against bronchoconstriction for a similar period [3]. Both improve ASTHMA control (when given twice daily) compared with regular treatment with short-acting β_2-agonists four times daily, and are well tolerated. TOLERANCE to the bronchodilator effect of formoterol and the bronchoprotective effects of formoterol and salmeterol have been demonstrated, but the resulting loss of protection is small, does not appear to be progressive and is of doubtful clinical significance. While both drugs have a similar duration of effect in clinical studies, there are differences. Formoterol has a more rapid onset of action and is an almost full agonist, whereas salmeterol is a partial agonist with a slower onset of action. These differences might confer a theoretical advantage for formoterol in more severe ASTHMA, whereas it may also make it more likely to induce TOLERANCE. However, no significant clinical differences between salmeterol and formoterol have been found in the treatment of patients with severe ASTHMA [6]. The rapid onset of action of formoterol means that it can be used as a reliever, whereas salmeterol can not [7].

In COPD, LABA are effective bronchodilators that may be used alone or in combination with anticholinergics or inhaled corticosteroids (ICS) [8]. LABA improve symptoms and exercise TOLERANCE by reducing air trapping, and also reduce exacerbations. In ASTHMA patients LABA should never be used alone as they do not treat the underlying chronic INFLAMMATION, so should always be used in combination with ICS (preferably in a fixed combination inhaler). LABA are an effective add-on therapy to ICS and are more effective than increasing the dose of ICS when ASTHMA is not controlled at low doses.

Combination inhalers

Combination inhalers that contain a LABA and a corticosteroid (fluticasone propionate/salmeterol, budesonide/formoterol, beclomethasone dipropionate/formoterol) are now widely used in the treat-

ment of ASTHMA and COPD. In ASTHMA there is a strong scientific rationale for combining a LABA with a corticosteroid as these treatments have complementary actions, and may also interact positively with the corticosteroids enhancing the effect of the LABA and the LABA potentiating the effect of the corticosteroid [9]. The combination inhaler is more convenient for patients, simplifies therapy and improves compliance with inhaled corticosteroids as the patients perceive clinical benefit, but there may be an additional advantage as delivering the two drugs in the same inhaler ensures that they are delivered simultaneously to the same cells in the airways, allowing the beneficial molecular interactions between LABA and corticosteroids to occur. It is likely that these inhalers will become the preferred therapy for all patients with persistent ASTHMA. These combination inhalers are also more effective in COPD patients than LABA and ICS alone, but the mechanisms accounting for this beneficial interaction are less well understood than in patients with ASTHMA.

Recently a combination inhaler that contains formoterol and budesonide has been shown to be more effective as a reliever than either terbutaline or formoterol alone, suggesting that the inhaled corticosteroids may also be contributing to the benefit [10]. This may make it possible to control ASTHMA with a single inhaler both for maintenance and relief of symptoms (see below).

Stereoselective β_2-agonists

Albuterol is a racemic mixture of active *R*- and inactive *S*-isomers. Animal studies have suggested that the *S*-isomer may increase airway responsiveness, providing a rationale for the development of *R*-albuterol (levalbuterol). Although the *R*-isomer is more potent than racemic *RS*-albuterol in some studies, careful dose-responses show no advantage in terms of EFFICACY and no evidence that the *S*-albuterol is detrimental in asthmatic patients [11]. As levalbuterol is more expensive than normally used racemic albuterol, this therapy has no clear clinical advantage [12]. Stereoselective formoterol (*R,R*-formoterol, arformoterol) has now been developed as a nebulized solution, but appears to offer no clinical advantage over racemic formoterol [13].

β_2-receptor polymorphisms

Several single nucleotide polymorphisms and haplotypes of the human β_2-adrenergic RECEPTOR gene (*ADRβ2*), which affect the structure of β_2-RECEPTORS, have been described. The common variants are $Gly^{16}Arg$ and $Gln^{27}Glu$, which have *in vitro* effects on RECEPTOR desensitization, but clinical studies have shown inconsistent effects on the bronchodilator responses to short- and long-acting β_2-agonists [14]. Some studies have shown that patients with the common homozygous $Arg^{16}Arg$ variant have more frequent adverse effects and a poorer response to short-acting β_2-agonist than heterozygotes or $Gly^{16}Gly$ homozygotes, but overall these differences are small and there appears to be no clinical value in measuring *ADRβ2* genotype. No differences have been found with responses to LABA between these genotypes [15].

Side effects

Unwanted effects are dose-related and are due to stimulation of extra-pulmonary β-RECEPTORS (Tab. 2). Side effects are not common with inhaled therapy, but more common with oral or especially intravenous administration.

- Muscle tremor is due to stimulation of β_2-RECEPTORS in skeletal muscle, and is the commonest side effect. It may be more troublesome with elderly patients and is a greater problem in COPD patients.
- Tachycardia and palpitations are due to reflex cardiac stimulation secondary to peripheral vasodilatation, from direct stimulation of atrial β_2-RECEPTORS (human heart has a relatively high proportion of β_2-RECEPTORS), and possibly also from stimulation of myocardial β_1-RECEPTORS as the doses of β_2-agonist are increased. These side effects tend to disappear with continued use of the drug, reflecting the development of TOLERANCE. There is a dose-related prolongation of the corrected QT interval (QTc).
- Metabolic effects (increase in free fatty acid, insulin, glucose, pyruvate and lactate) are usually seen only after large systemic doses.

Table 2. Side effects of β_2-agonists

Muscle tremor (direct effect on skeletal muscle β_2-receptors)
Tachycardia (direct effect on atrial β_2-receptors, reflex effect from increased peripheral vasodilatation *via* β_2-receptors)
Hypokalemia (direct effect on skeletal muscle uptake of K^+ *via* β_2-receptors)
Restlessness
Hypoxemia (increased V/Q mismatch due to pulmonary vasodilatation)
Decreased cholinergic neurotransmission
Decreased cough
No effect on chronic inflammation

- Hypokalemia is a potentially more serious side effect. This is due to β_2-receptor stimulation of potassium entry into skeletal muscle, which may be secondary to a rise in insulin secretion. Hypokalemia might be serious in the presence of hypoxia, as in acute asthma, when there may be a predisposition to cardiac dysrrhythmias. In practice, however, no significant arrhythmias after nebulized β_2-agonist have been reported in acute asthma or COPD patients.
- Ventilation-perfusion (V/Q) mismatching can be caused by pulmonary vasodilatation in blood vessels previously constricted by hypoxia, resulting in the shunting of blood to poorly ventilated areas and a fall in arterial oxygen tension. Although, in practice, the effect of β_2-agonists on $P_{A_{O_2}}$ is usually very small (<5 mm Hg fall), it is occasionally large in severe COPD, although it may be prevented by giving additional inspired oxygen.

Tolerance

Continuous treatment with an agonist often leads to tolerance (desensitization, subsensitivity), which may be due to down-regulation of the receptor. For this reason there have been many studies of bronchial β-receptor function after prolonged therapy with β_2-agonists. Tolerance of non-airway β_2-receptor-mediated responses, such as tremor and cardiovascular and metabolic responses, is readily induced in normal and asthmatic subjects. Tolerance of human airway smooth muscle to β_2-agonists *in vitro* has been demonstrated, although the concentration of agonist necessary is high and the degree of desensitization is variable. Animal studies suggest that airway smooth muscle β_2-receptors may be more resistant to desensitization that β_2-receptors elsewhere due to a high receptor reserve. In normal subjects, bronchodilator tolerance has been demonstrated in some studies after high-dose inhaled albuterol, but not in others. In asthmatic patients, tolerance to the bronchodilator effects of β_2-agonists has not usually been found. However, tolerance develops to the bronchoprotective effects of β_2-agonists and is more marked with indirect bronchoconstrictors, such as adenosine, allergen and exercise (that activate mast cells), than with direct bronchoconstrictors, such as histamine and methacholine. The reason for the relative resistance of airway smooth muscle β_2-receptors to desensitization remains uncertain, but may reflect the fact that there is a large receptor reserve, so that >90% of β_2-receptors may be lost without any reduction in the relaxation response. The high level of β_2-receptor gene expression in airway smooth muscle compared to peripheral lung may also contribute to the resistance to tolerance since there is likely to be a high rate of β-receptor synthesis. In addition, the expression of the enzyme G protein receptor kinase-2 (GRK2), which phosphorylates and inactivates occupied β_2-receptors, is very low in airway smooth muscle [16]. By contrast, there is no receptor reserve in inflammatory cells and GRK2 expression is high, so that indirect effects of β_2-agonists are more readily lost and tolerance to β_2-agonists rapidly develops.

Experimental studies have shown that corticosteroids prevent the development of tolerance in airway smooth muscle, and prevent and reverse the fall in pulmonary β-receptor density [17]. However, ICS fail to prevent the tolerance to the bronchoprotective effect of inhaled β_2-agonists, possibly because

they do not reach airway smooth muscle in a high enough concentration.

Long-term safety

Because of a possible relationship between adrenergic drug therapy and the rise in ASTHMA deaths in several countries during the early 1960s, doubts were cast on the long-term safety of β-agonists. A causal relationship between β-agonist use and mortality has never been firmly established, although in retrospective studies this would not be possible. A particular β_2-agonist, fenoterol, was linked to the rise in ASTHMA deaths in New Zealand in the early 1990s since significantly more of the fatal cases were prescribed fenoterol than the case-matched control patients. This association was strengthened by two subsequent studies and once fenoterol ceased to be available the ASTHMA mortality fell dramatically. An epidemiological study examined the links between drugs prescribed for ASTHMA and death or near death from ASTHMA attacks, based on computerized records of prescriptions. There was a marked increase in the risk of death with high doses of all inhaled β_2-agonists [18]. The risk was greater with fenoterol, but when the dose was adjusted to the equivalent dose for albuterol there was no significant difference in the risk for these two drugs. The link between high β_2-agonist usage and increased ASTHMA mortality does not prove a causal association, since patients with more severe and poorly controlled ASTHMA, and who are therefore more likely to have an increased risk of fatal attacks, are more likely to be using higher doses of β_2-agonist inhalers and less likely to be using effective anti-inflammatory treatment. Indeed, in the patients who used regular inhaled steroids there was a significant reduction in risk of death [19].

Regular use of inhaled β_2-agonists has also been linked to increased ASTHMA morbidity. Regular use of fenoterol was associated with worse ASTHMA control and a small increase in airway hyperresponsiveness compared to patients using fenoterol 'on demand' for symptom control over a 6-month period [20]. However, this was not found in a study with regular albuterol [21]. There is some evidence that regular inhaled β_2-agonists may increase allergen-induced ASTHMA and sputum eosinophilia [22]. A possible mechanism is that β_2-agonists activate phospholipase C *via* coupling through Gq, resulting in augmentation of the bronchoconstrictor responses to cholinergic agonists and mediators [23]. Short-acting inhaled β_2-agonists should only be used 'on demand' for symptom control and if they are required frequently (more than three times weekly) then an ICS is needed.

The safety of LABA in ASTHMA remains controversial. A large study of the safety of salmeterol showed an excess of respiratory deaths and near deaths in patients prescribed salmeterol but these deaths occurred mainly in African Americans living in inner cities who were not taking ICS [24]. Similar data have also raised concerns about formoterol. This may be predictable since LABA do not treat the underlying chronic INFLAMMATION of ASTHMA. However, concomitant treatment with ICS appears to obviate any risk so it is recommended that LABA should only be used when ICS are also prescribed (preferably in the form of a combination inhaler so that the LABA can never be taken without the inhaled corticosteroids) [25,26]. There are no safety concerns with LABA use in COPD and no adverse effects were reported in a large study over 3 years in COPD patients and in several other studies [27,28].

Future developments

β_2-Agonists will continue to be the bronchodilators of choice for ASTHMA in the foreseeable future, as they are effective in all patients and have few or no side effects when used in low doses. It would be difficult to find a bronchodilator that improves on the EFFICACY and safety of inhaled β_2-agonists. Although some concerns have been expressed about the long-term effects of short-acting inhaled β_2-agonists, when used as required for symptom control, inhaled β_2-agonists are safe. In patients who are using large doses, their ASTHMA must be assessed and appropriate controller medication used. LABA are very useful for long-term control in ASTHMA and COPD. In ASTHMA patients, LABA should only be used if the patient is receiving concomitant ICS.

There is little advantage to be gained by improving β_2-RECEPTOR selectivity, since most of the side effects of β-agonists are due to β_2-RECEPTOR stimulation (muscle tremor, tachycardia, hypokalemia). Several once-daily inhaled β_2-agonists, such as indacaterol, carmoterol and vilanterol, are now in clinical development [4].

Theophylline

Methylxanthines, such as theophylline, which are related to caffeine, have been used in the treatment of ASTHMA since 1930 and theophylline is still widely used in developing countries because it is inexpensive. Theophylline became more useful with the availability of rapid plasma assays and the introduction of reliable slow-release preparations. However, the frequency of side effects and the relative low EFFICACY of theophylline have recently led to reduced usage in many countries, since inhaled β_2-agonists are far more effective as bronchodilators and ICS have a greater anti-inflammatory effect. In patients with severe ASTHMA and COPD it still remains a very useful drug, however. There is increasing evidence that theophylline has anti-inflammatory or immunomodulatory effects and may enhance the anti-inflammatory effects of corticosteroids [29].

Chemistry

Theophylline is a methylxanthine similar in structure to the common dietary xanthines caffeine and theobromine. Several substituted derivatives have been synthesized but none has any advantage over theophylline, apart from the 3-propyl derivative, enprofylline, which is more potent as a bronchodilator and may have fewer toxic effects as it does not antagonize adenosine RECEPTORS. Many salts of theophylline have also been marketed, the most common being aminophylline, which is the ethylenediamine salt, used to increase its solubility at neutral pH. Other salts, such as choline theophyllinate, do not have any advantage and others, such as acepifylline, are virtually inactive, so that theophylline remains the major methylxanthine in clinical use. Doxofylline [7-(1,3-dioxalan-2-ylmethyl)theophylline] is a novel methylxanthine with a dioxalane group at position 7, and is available in some countries [30]. It has a similar inhibitory effect on PHOSPHODIESTERASES to theophylline but is less active as an adenosine antagonist so has a better side effect profile.

Mode of action

The mechanism of action of theophylline is still uncertain. In addition to its bronchodilator action, theophylline has many non-bronchodilator effects that may be relevant to its beneficial effects in ASTHMA and COPD. However, many of these molecular mechanisms are seen only at high concentrations that exceed the therapeutic range.

Non-bronchodilator effects

Theophylline has clinical benefit in ASTHMA and COPD at plasma concentrations <10 mg/L, which are unlikely to be explained by its bronchodilator action. There is increasing evidence that theophylline has anti-inflammatory effects in ASTHMA [29]. Chronic oral treatment with theophylline inhibits the late response to inhaled allergen and a reduced infiltration of EOSINOPHILS and $CD4^+$ LYMPHOCYTES into the airways after allergen challenge. In patients with mild ASTHMA, low doses of theophylline (mean plasma concentration ~5 mg/L) reduce the numbers of EOSINOPHILS in bronchial biopsies, bronchoalveolar lavage and induced sputum [31], whereas in severe ASTHMA withdrawal of theophylline results in increased numbers of activated $CD4^+$ cells and EOSINOPHILS in bronchial biopsies [32]. In patients with COPD, theophylline reduces the total number and proportion of NEUTROPHILS in induced sputum, the concentration of INTERLEUKIN-8 and neutrophil chemotactic responses, suggesting that it may have an anti-inflammatory effect [33]. Theophylline withdrawal in COPD patients results in worsening of disease. *In vitro* theophylline is able to increase responsiveness to corticosteroids and to reverse corticosteroid resistance in COPD cells [34].

Molecular mechanisms of action

Several molecular MECHANISMS OF ACTION have been proposed for theophylline (Tab. 3).

Inhibition of phosphodiesterases

PHOSPHODIESTERASES (PDE) break down cyclic nucleotides in the cell, thereby leading to a decrease in intracellular cAMP and cyclic guanosine 3'5 monophosphate (cGMP) concentrations (Fig. 3). Theophylline is a non-selective PDE inhibitor, but the degree of inhibition is relatively minimal at concentrations of theophylline within the therapeutic range. PDE inhibition almost certainly accounts for the bronchodilator action of theophylline, but this is unlikely to account for the non-bronchodilator effects of theophylline that are seen at lower concentrations. Inhibition of PDE should lead to synergistic interaction with β-agonists through an increase in cAMP, but this has not been convincingly demonstrated in clinical studies. Several isoenzyme families of PDE have now been recognized and those important in smooth muscle relaxation include PDE3, PDE4 and PDE5. Theophylline is a weak inhibitor of all PDE isoenzymes.

Adenosine receptor antagonism

Theophylline antagonizes adenosine RECEPTORS at therapeutic concentrations. Of particular importance may be the adenosine A_{2B} RECEPTOR on MAST CELLS, which is activated by adenosine in asthmatic patients [35]. *In vitro* adenosine has little direct effect on human airway smooth muscle, but causes bronchoconstriction in airways from asthmatic patients by releasing HISTAMINE and LEUKOTRIENES. Adenosine antagonism is unlikely to account for the anti-inflammatory effects of theophylline but may be responsible for serious side effects, including cardiac arrhythmias and seizures through the antagonism of A_1 RECEPTORS.

TABLE 3. MECHANISMS OF ACTION OF THEOPHYLLINE

Phosphodiesterase inhibition (non-selective)
Adenosine receptor antagonism (A_1, A_{2A}, A_{2B}-receptors)
Increased interleukin-10 release
Stimulation of catecholamine (adrenaline) release
Mediator inhibition (prostaglandins, tumor necrosis factor-α)
Inhibition of intracellular calcium release
Inhibition of nuclear factor-κB (↓ nuclear translocation)
Increased apoptosis
↑ Histone deacetylase activity (↑ efficacy of corticosteroids)

Interleukin-10 release

IL-10 has a broad spectrum of anti-inflammatory effects and there is evidence that its secretion is reduced in ASTHMA. IL-10 release is increased by theophylline and this effect may be mediated *via* PDE inhibition, although this has not been seen at the low doses that are effective in ASTHMA [36].

Effects on gene transcription

Theophylline prevents the translocation of the proinflammatory transcription factor NUCLEAR FACTOR-κB (NF-κB) into the nucleus, thus potentially reducing the expression of inflammatory genes in ASTHMA and COPD. Inhibition of NF-κB appears to be due to a protective effect against the degradation of the inhibitory protein I-κBα, so that nuclear translocation of activated NF-κB is prevented [37]. However, these effects are seen at high concentrations and may be mediated by inhibition of PDE.

Effects on apoptosis

Prolonged survival of GRANULOCYTES due to a reduction in APOPTOSIS may be important in perpetuating chronic INFLAMMATION in ASTHMA (EOSINOPHILS) and COPD (NEUTROPHILS). Theophylline promotes APOPTOSIS in EOSINOPHILS and NEUTROPHILS *in vitro*. This is associated with a reduction in the anti-apoptotic protein Bcl-2 [38]. This effect is not mediated *via* PDE inhibition, but in NEUTROPHILS may be mediated by antagonism of adenosine A_{2A} RECEPTORS [39]. Theophylline also induces APOPTOSIS of T LYMPHOCYTES, thus reducing their survival and this effect appears to be mediated *via* PDE inhibition [40].

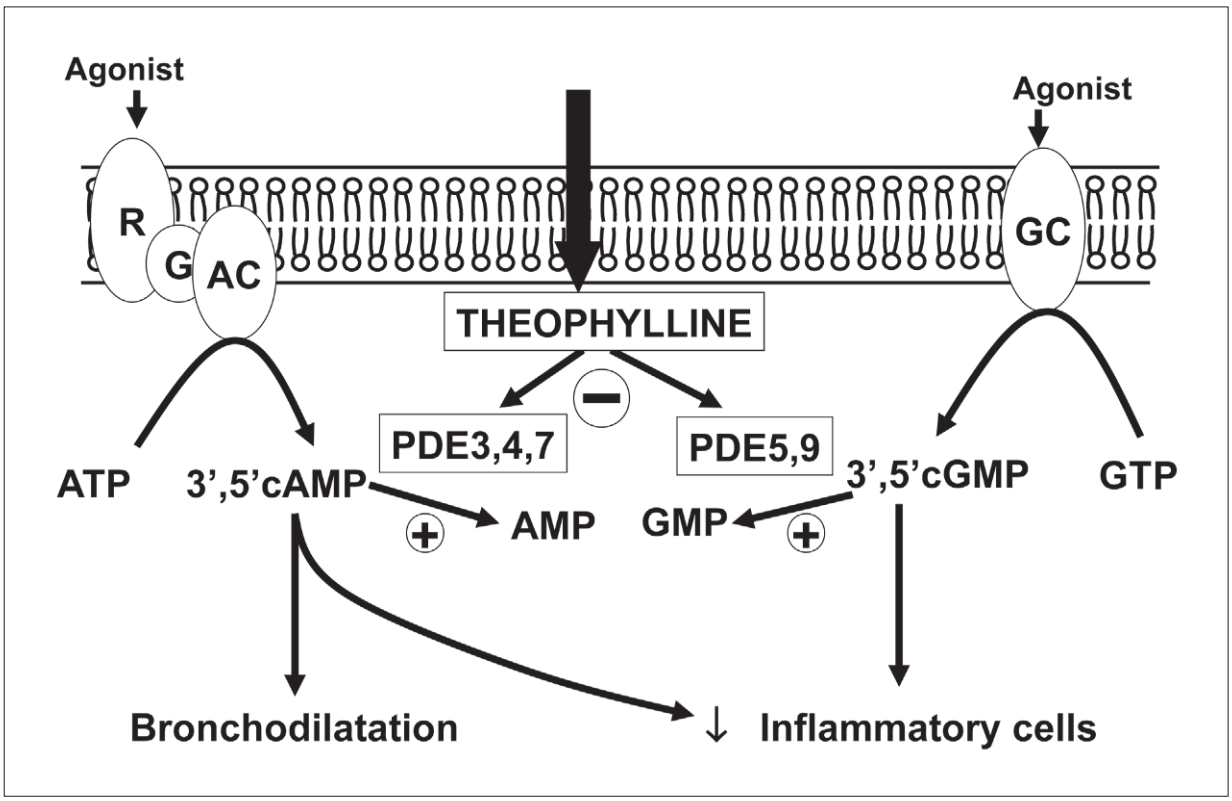

FIGURE 3

The inhibitory effect of theophylline on phosphodiesterases (PDE) may result in bronchodilatation and inhibition of inflammatory cells. ATP, adenosine triphosphate; AMP, adenosine monophosphate; PKA, protein kinase A; GTP, guanosine triphosphate; GMP, guanosine monophosphate; PKG, protein kinase G.

Histone deacetylase activation

Recruitment of HISTONE DEACETYLASE-2 (HDAC2) by glucocorticoid RECEPTORS switches off inflammatory genes (see below) (Fig. 4). Theophylline is an activator of HDAC at therapeutic concentrations, thus enhancing the anti-inflammatory effects of corticosteroids [34, 41]. This mechanism is independent of PDE inhibition or adenosine antagonism and appears to be mediated by inhibition of phosphoinositide-3-kinase-δ, which is activated by oxidative stress [42, 43]. The anti-inflammatory effects of theophylline are inhibited by an HDAC inhibitor trichostatin A. Low doses of theophylline increase HDAC activity in bronchial biopsies of asthmatic patients and correlate with the reduction in eosinophil numbers in the biopsy.

Other effects

Several other effects of theophylline have been described, including an increase in circulating CATECHOLAMINES, inhibition of calcium influx into inflammatory cells, inhibition of PROSTAGLANDIN effects, and antagonism of TUMOR NECROSIS FACTOR-α. These effects are generally seen only at high concentrations of theophylline that are above the therapeutic range in ASTHMA and are therefore unlikely to contribute to the anti-inflammatory actions of theophylline.

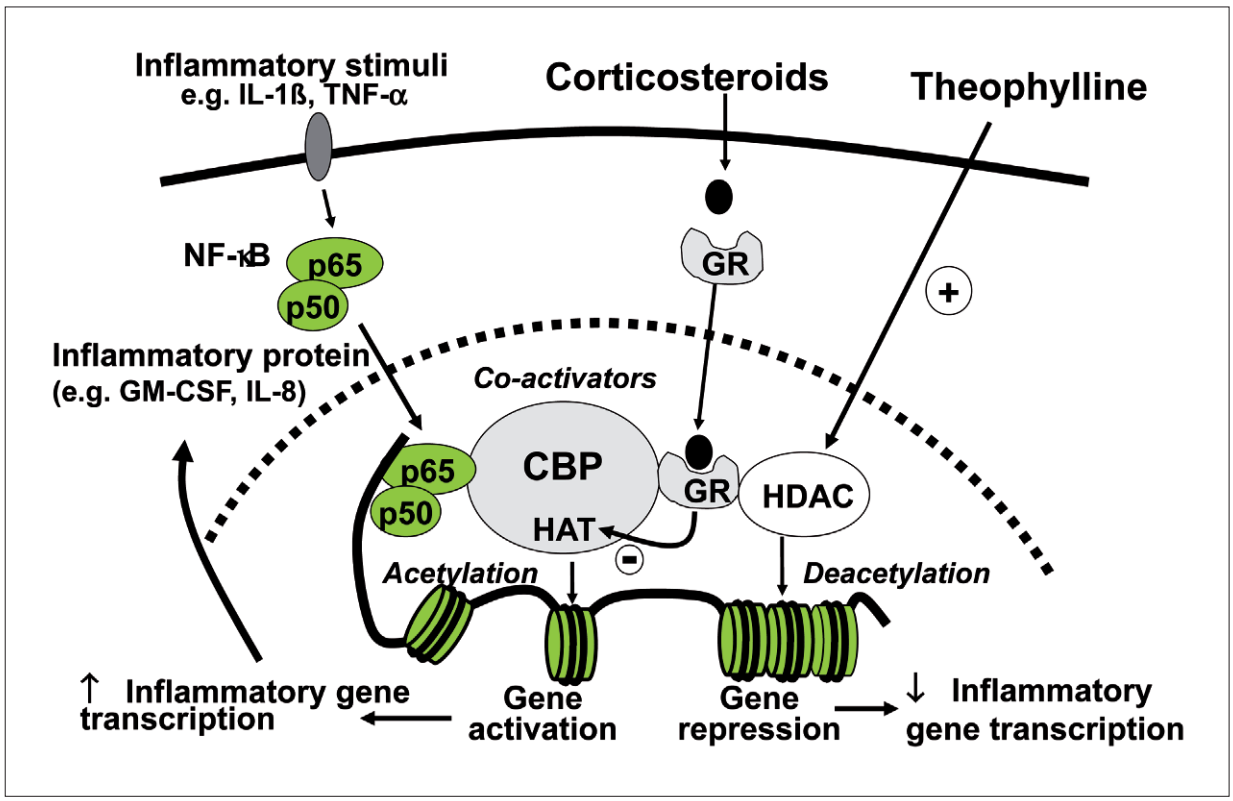

FIGURE 4

Theophylline directly activates histone deacetylases (HDACs), which deacetylate core histones that have been acetylated by the histone acetyltransferase (HAT) activity of co-activators, such as CREB-binding protein (CBP). This results in suppression of inflammatory genes and proteins, such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-8 (IL-8) that have been switched on by proinflammatory transcription factors, such as nuclear factor-κB (NF-κB). Corticosteroids also activate HDACs, but through a different mechanism, resulting in the recruitment of HDACs to the activated transcriptional complex via activation of the glucocorticoid receptors (GR), which function as a molecular bridge. This predicts that theophylline and corticosteroids may have a synergistic effect in repressing inflammatory gene expression.

Pharmacokinetics and metabolism

There is a close relationship between improvement in airway function and serum theophylline concentration. Below plasma levels of 10 mg/L therapeutic effects (at least in terms of bronchodilatation) are small, and above 25 mg/L additional benefits are outweighed by side effects, so that the therapeutic range was usually taken as 10–20 mg/L (55–110 μM). However, theophylline has anti-ASTHMA effects other than bronchodilatation, and these are seen below 10 mg/L so the therapeutic range is now taken as 5–15 mg/L. The dose of theophylline required to give these therapeutic concentrations varies between subjects, largely because of differences in CLEARANCE of the drug. In addition, there may be differences in bronchodilator response to theophylline and with acute bronchoconstriction higher concentrations

may be required to produce bronchodilatation. Theophylline is rapidly and completely absorbed, but there are large inter-individual variations in CLEARANCE, due to differences in hepatic metabolism (Tab. 4). Theophylline is metabolized in the liver by the cytochrome P450 microsomal enzyme system (mainly CYP1A2), and a large number of factors may influence hepatic metabolism [44].

Increased CLEARANCE is seen in children (1–16 years), and in cigarette and marijuana smokers due to induction of CYP1A2. Concurrent administration of phenytoin, phenobarbitone and rifampicin increases activity of P450 isoenzymes (especially CYP1A2), resulting in increased metabolic breakdown, so that higher doses may be required.

Reduced CLEARANCE is found in liver disease, pneumonia and heart failure, and doses need to be reduced to half and plasma levels monitored carefully. Decreased CLEARANCE is also seen with certain drugs, including erythromycin, certain quinolone antibiotics (ciprofloxacin, but not ofloxacin), allopurinol, cimetidine (but not ranitidine), fluoxamine, the 5'-LIPOXYGENASE inhibitor zileuton and the LEUKOTRIENE RECEPTOR antagonist zafirlukast, all of which interfere with cytochrome P450 function. Thus, if a patient on maintenance theophylline requires a course of erythromycin, the dose of theophylline should be halved. Viral infections and vaccination may also reduce CLEARANCE, and this may be particularly important in children. Because of these variations in CLEARANCE, individualization of theophylline dosage is required and plasma concentrations should be measured 4 hours after the last dose with slow-release preparations when steady state has usually been achieved. There is no significant circadian variation in theophylline metabolism, although there may be delayed absorption at night, which may relate to the supine posture.

Table 4. Factors affecting clearance of theophylline

Increased clearance
Enzyme induction (rifampicin, phenobarbitone, ethanol)
Smoking (tobacco, marijuana)
High protein, low carbohydrate diet
Barbecued meat
Childhood
Decreased clearance
Enzyme inhibition (cimetidine, erythromycin, ciprofloxacin, allopurinol, zileuton, zafirlukast)
Congestive heart failure
Liver disease
Pneumonia
Viral infection and vaccination
High carbohydrate diet
Old age

Routes of administration

Intravenous aminophylline has been used for many years in the treatment of acute severe ASTHMA. The recommended dose is now 6 mg/kg given intravenously over 20–30 minutes, followed by a maintenance dose of 0.5 mg/kg/hour. If the patient is already taking theophylline, or there are any factors that decrease CLEARANCE, these doses should be halved and the plasma level checked more frequently. Nebulized β_2-agonists are now preferred as bronchodilators for acute exacerbations of ASTHMA and COPD.

Oral plain theophylline tablets or elixir, which are rapidly absorbed, give wide fluctuations in plasma levels and are not recommended. Several sustained-release preparations are now available, which are absorbed at a constant rate and provide steady plasma concentrations over a 12–24 hour period. Although there are differences between preparations, these are relatively minor and of no clinical significance. Both slow-release aminophylline and theophylline are available and are equally effective (although the ethylenediamine component of aminophylline has very occasionally been implicated in allergic reactions). For continuous treatment twice-daily therapy (approximately 8 mg/kg twice daily) is needed, although some preparations are designed for once-daily administration. For nocturnal ASTHMA, a single dose of slow-release theophylline at night is

often effective. Once optimal doses have been determined plasma concentrations usually remain stable, providing no factors which alter CLEARANCE change.

Aminophylline may be given as a suppository, but rectal absorption is unreliable and proctitis may occur, so is best avoided. Inhalation of theophylline is irritant and ineffective until therapeutic plasma concentrations are reached. Intramuscular injections of theophylline are very painful and should never be given.

Clinical use

In patients with acute ASTHMA intravenous aminophylline is less effective than nebulized β_2-agonists, and should therefore be reserved for those patients who fail to respond to β-agonists. Theophylline should not be added routinely to nebulized β_2-agonists since it does not increase the bronchodilator response and may only increase their side effects [45].

Theophylline has little or no effect on bronchomotor tone in normal airways, but reverses bronchoconstriction in asthmatic patients, although it is less effective than inhaled β_2-agonists and is more likely to have unwanted effects. Indeed, the role of theophylline in the management of ASTHMA and COPD has been questioned. There is good evidence that theophylline and β_2-agonists have additive effects, even if true synergy is not seen, and there is evidence that theophylline may provide an additional bronchodilator effect even when maximally effective doses of β_2-agonist have been given [46]. This means that, if adequate bronchodilatation is not achieved by a β-agonist alone, theophylline may be added to the maintenance therapy with benefit. Addition of low-dose theophylline to either high or low dose of inhaled corticosteroids in patients who are not adequately controlled provides better symptom control and lung function than doubling the dose of inhaled steroid [47–49], although it is less effective as an add-on therapy than an LABA [50]. Theophylline may be useful in patients with nocturnal ASTHMA, since slow-release preparations are able to provide therapeutic concentrations overnight, although it is less effective than an LABA [51]. Studies have also documented steroid-sparing effects of theophylline. Although theophylline is less effective than a β_2-agonist and corticosteroids, there are a minority of asthmatic patients who appear to derive unexpected benefit, and even patients on oral steroids may show a deterioration in lung function when theophylline is withdrawn [32, 52]. Theophylline has been used as a controller in the management of mild persistent ASTHMA, although it is usually found to be less effective than low doses of inhaled corticosteroids [53]. Theophylline is currently a less preferred option than ICS, and is recommended as a second-line choice of controller in management of asthmatic patients. Although LABA are more effective as an add-on therapy at Steps 3 and 4 of the GINA (Global initiative for ASTHMA) guidelines, theophylline is considerably less expensive and may be the only affordable add-on treatment when the costs of medication are limiting [54].

Theophylline is still used as a bronchodilator in COPD, but inhaled anticholinergics and β_2-agonists are preferred [55]. Theophylline tends to be added to these inhaled bronchodilators for patients with more severe disease, and has been shown to give additional clinical improvement when added to a long-acting β_2-agonist [56]. Intravenous aminophylline is less effective in treating acute exacerbations than nebulized β_2-agonists, and has a much higher incidence of adverse effects [57, 58]. As in ASTHMA, patients with severe COPD deteriorate when theophylline is withdrawn from their treatment regime. A theoretical advantage of theophylline is that it its systemic administration may have effects on small airways, resulting in reduction of hyperinflation and thus a reduction in dyspnea.

Side effects

Unwanted effects of theophylline are usually related to plasma concentration and tend to occur when plasma levels exceed 15 mg/L. However, many patients develop side effects even at low plasma concentrations. To some extent side effects may be reduced by gradually increasing the dose until therapeutic concentrations are achieved.

The commonest side effects are headache, nausea and vomiting (due to inhibition of PDE4), abdominal discomfort and restlessness (Tab. 5). There may also

Table 5. Side effects of theophylline

	Mechanism
Nausea and vomiting	PDE4 inhibition
Headaches	PDE4 inhibition
Gastric discomfort	PDE4 inhibition
Diuresis	A_1 receptor antagonism
Cardiac arrhythmias	A_1 receptor antagonism PDE3 inhibition
Epileptic seizures	A_1 receptor antagonism

be increased acid secretion and diuresis (due to inhibition of adenosine A_1 receptors). Theophylline may lead to behavioral disturbance and learning difficulties in school children, although it is difficult to design adequate controls for such studies.

At high concentrations cardiac arrhythmias may occur as a consequence of PDE3 inhibition and adenosine A_1 receptor antagonism, and at very high concentrations seizures may occur (due to central A_1 receptor antagonism). Use of low doses of theophylline, giving plasma concentrations of 5–10 mg/L largely avoids side effects and drug interactions, and makes it unnecessary to monitor plasma concentrations (unless checking for compliance).

Future developments

Theophylline use has been declining, partly because of the problems with side effects, but mainly because more effective therapy with inhaled corticosteroids has been introduced. Oral theophylline is still a very useful treatment in some patients with difficult asthma, and appears to have effects beyond those provided by steroids. Rapid release theophylline preparations are cheap and are the only affordable anti-asthma medication in some developing countries. There is increasing evidence that theophylline has some anti-asthma effect at doses that are lower than those needed for bronchodilatation and plasma levels of 5–15 mg/L are recommended, instead of the previously recommended 10–20 mg/L. Adding a low dose of theophylline gives better control of asthma than doubling the dose of ICS in patients who are not adequately controlled, and is a less expensive alternative add-on therapy than an LABA or anti-leukotriene.

Now that the molecular mechanisms for the anti-inflammatory effects of theophylline are better understood, there is a strong scientific rationale for combining low dose theophylline with ICS, particularly in patients with more severe asthma and in COPD. Theophylline reverses corticosteroid resistance in COPD cells by restoring HDAC2 to normal levels [41] and accelerates the recovery from acute exacerbations of COPD through this mechanism [59]. In COPD, low dose theophylline is the first drug to demonstrate clear anti-inflammatory effects, and thus it may even have a role in preventing progression of the disease [33].

Anticholinergics

Datura plants, which contain the muscarinic antagonist strammonium, were smoked for relief of asthma two centuries ago. Atropine, a related naturally occurring compound, was also introduced for treating asthma but these compounds gave side effects, particularly drying of secretions, so less soluble quaternary compounds, such as atropine methylnitrate and ipratropium bromide, were developed. These compounds are topically active and are not significantly absorbed from the respiratory or gastrointestinal tracts.

Mode of action

Anticholinergics are antagonists of muscarinic receptors and in therapeutic use have no other significant pharmacological effects. In animals and man there is a small degree of resting bronchomotor tone, which is probably due to tonic vagal nerve impulses that release acetylcholine in the vicinity of airway smooth muscle, since it can be blocked by anticholinergic drugs. Acetylcholine may also be released from other airway cells, including epithelial cells

[60, 61]. The synthesis of ACETYLCHOLINE in epithelial cells is increased by inflammatory stimuli (such as TNF-α), which increases the expression of choline acetyltransferase and this could contribute to cholinergic effects in airway diseases. Since muscarinic RECEPTORS are expressed in smooth muscle of small airways that do not appear to be innervated by cholinergic nerves, this might be important as a mechanism of cholinergic narrowing in peripheral airways that could be relevant in COPD.

Cholinergic pathways may play an important role in regulating acute bronchomotor responses in animals, and there are a wide variety of mechanical, chemical and immunological stimuli that elicit reflex bronchoconstriction *via* vagal pathways. This suggested that cholinergic mechanisms might underlie airway hyperresponsiveness and acute bronchoconstrictor responses in ASTHMA, with the implication that anticholinergic drugs would be effective bronchodilators. While these drugs may afford protection against acute challenge by sulfur dioxide, inert dusts, cold air and emotional factors, they are less effective against antigen challenge, exercise and fog. This is not surprising as anticholinergic drugs only inhibit reflex cholinergic bronchoconstriction and have no blocking effect on the direct effects of inflammatory mediators, such as HISTAMINE and LEUKOTRIENES, on bronchial smooth muscle. Furthermore, cholinergic antagonists probably have little or no effect on MAST CELLS, microvascular leak or the chronic inflammatory response.

Theoretically, anticholinergics may reduce airway mucus secretion and reduce mucus CLEARANCE, but this does not appear to happen in clinical studies. Oxitropium bromide in high doses reduces mucus hypersecretion in patients with COPD with chronic bronchitis.

Clinical use

In asthmatic patients anticholinergic drugs are less effective as bronchodilators than β_2-agonists and offer less efficient protection against various bronchial challenges. These drugs may be more effective in older patients with ASTHMA in whom there is an element of fixed airway obstruction. Anticholinergics are currently used as an additional bronchodilator in asthmatic patients not controlled on an LABA. Nebulized anticholinergic drugs are effective in acute severe ASTHMA, although they are less effective than β_2-agonists in this situation. Nevertheless, in the acute and chronic treatment of ASTHMA, anticholinergic drugs may have an additive effect with β_2-agonists and should therefore be considered when control of ASTHMA is not adequate with nebulized β_2-agonists, particularly if there are problems with theophylline, or inhaled β_2-agonists give troublesome tremor in elderly patients.

In COPD, anticholinergic drugs may be as effective as, or even superior to β_2-agonists. Their relatively greater effect in COPD than in ASTHMA may be explained by an inhibitory effect on vagal tone, which, while not necessarily being increased in COPD, may be the only reversible element of airway obstruction, which is exaggerated by geometric factors in the narrowed airways of COPD patients (Fig. 5). By contrast, β_2-agonists acting as functional antagonists reverse all bronchoconstrictor mechanisms, including the bronchoconstrictor effect of multiple inflammatory mediators in ASTHMA.

Therapeutic choices

Ipratropium bromide is available as a pMDI and nebulized preparation. The onset of bronchodilatation is relatively slow and is usually maximal 30–60 minutes after inhalation, but may persist for 6–8 hours. It is usually given by MDI three to four times daily on a regular basis, rather than intermittently for symptom relief, in view of its slow onset of action.

Oxitropium bromide is a quaternary anticholinergic bronchodilator, and is similar to ipratropium bromide in terms of RECEPTOR blockade. It is available in higher doses by inhalation and may therefore have a more prolonged effect. Thus, it may be useful in some patients with nocturnal ASTHMA.

Combination inhalers of an anticholinergic and β_2-agonist, such as ipratropium/albuterol, are popular, particularly in patients with COPD. Several studies have demonstrated additive effects of these two drugs, thus providing an advantage over increasing the dose of β_2-agonist in patients who have side effects.

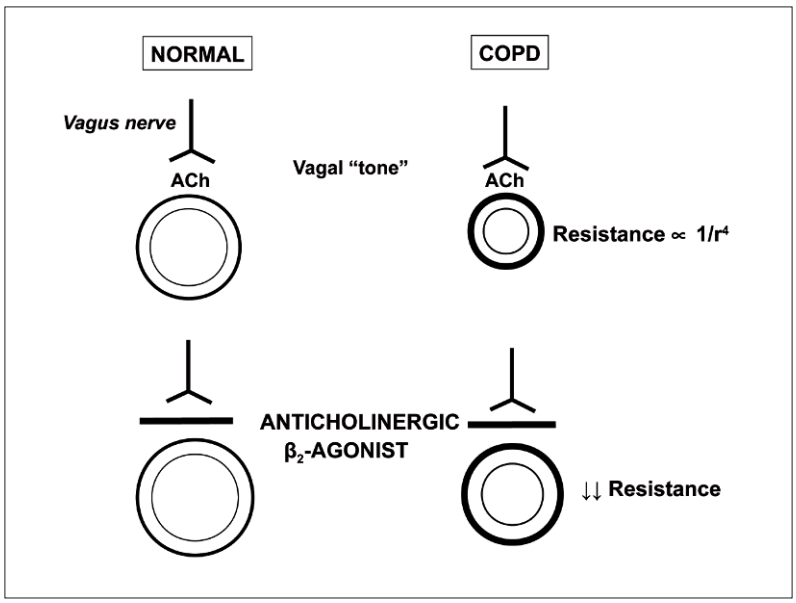

Figure 5
Anticholinergic drugs inhibit vagally mediated airway tone leading to bronchodilatation. This effect is small in normal airways but is greater in airways of patients with chronic obstructive pulmonary disease (COPD), which are structurally narrowed as airway resistance (R) is inversely related to the fourth power of the radius (r).

Tiotropium bromide is a long-acting anticholinergic drug that is suitable for once-daily dosing as a dry powder inhaler or *via* a soft mist mini-nebulizer device [62]. It binds with equal AFFINITY to all muscarinic RECEPTOR subtypes but dissociates very slowly from M_3 and M_1 RECEPTORS, giving it a degree of kinetic RECEPTOR selectivity for these RECEPTORS compared to M_2 RECEPTORS, from which it dissociates more rapidly. It is now the bronchodilator of choice in COPD patients. It is a more effective bronchodilator in patients with COPD than ipratropium four times daily, without any loss of EFFICACY over a 1 year-treatment period. Tiotropium reduces air trapping and improves exercise TOLERANCE. Over a 4-year period tiotropium improves lung function and health status and reduces exacerbations, although there is no effect on disease progression [63].

Side effects

Inhaled anticholinergic drugs are usually well tolerated and there is no evidence for any decline in responsiveness with continued use. On stopping inhaled anticholinergics a small rebound increase in responsiveness has been described, but the clinical relevance of this is uncertain. Atropine has side effects that are dose-related, and are due to cholinergic antagonism in other systems, which may lead to dryness of the mouth, blurred vision and urinary retention. Systemic side effects after ipratropium bromide and tiotropium bromide are very uncommon because there is virtually no systemic absorption. Because cholinergic agonists stimulate mucus secretion there have been several studies of mucus secretion with anticholinergic drugs as there has been concern that they may reduce secretion and lead to more viscous mucus. Atropine reduces mucociliary CLEARANCE in normal subjects and in patients with ASTHMA and chronic bronchitis, but ipratropium bromide, even in high doses, has no detectable effect in either normal subjects or in patients with airway disease. A significant unwanted effect is the unpleasant bitter taste of inhaled ipratropium, which may contribute to poor compliance with this drug. Nebulized ipratropium bromide may precipitate glaucoma in elderly patients due to a direct effect of the nebulized drug on the eye. This may be prevented by nebulization with a mouthpiece rather than a face mask. Reports of paradoxical bronchoconstriction with ipratropium bromide, particularly when given by nebulizer, were largely explained by the hypotonicity of the nebulizer solution and by antibacterial additives, such as ben-

zalkonium chloride and EDTA. Nebulizer solutions free of these problems are less likely to cause bronchoconstriction. This problem has not been described with tiotropium. Occasionally, bronchoconstriction may occur with ipratropium bromide given by MDI. It is possible that this is due to blockade of pre-junctional M_2 RECEPTORS on airway cholinergic nerves that normally inhibit ACETYLCHOLINE release.

Tiotropium is well tolerated and the only side effect of note is dryness of the mouth in 10–15% of patients, but this usually disappears and it rarely a reason for discontinuing therapy. Occasionally urinary retention is seen in elderly patients.

Future developments

Anticholinergics are the bronchodilators of choice in COPD and therefore have a growing market. Several other long-acting muscarinic antagonists (LAMA) are now in clinical development, including glycopyrrolate and aclidinium [64, 65]. Several combination inhalers of LAMA and LABA are also in development, as there is an additive effect between these classes of drug in COPD patients [66]. Dual action drugs that are both muscarinic antagonists and β_2-agonist (MABA) are also in clinical development [67]

Novel classes of bronchodilators

Several new classes of bronchodilator are under development, but it is difficult to envisage a more effective bronchodilator than an LABA for ASTHMA and long-acting anticholinergic for COPD. It has been difficult to find new classes of bronchodilator and several new potential drugs have often had problems with vasodilator side effects since they relax vascular smooth muscle to a greater extent than airway smooth muscle.

Magnesium sulfate

There is increasing evidence that magnesium sulfate is useful as an additional bronchodilator in patients with acute severe ASTHMA. Intravenous or nebulized magnesium sulfate benefit adults and children with severe exacerbations (FEV_1 <30% predicted), giving improvement in lung function when added to nebulized β_2-agonist, and a reduction in hospital admissions, although the effects are marginal [68]. The treatment is cheap and well tolerated. Side effects include flushing and nausea but are usually minor. It appears to act as a bronchodilator and may reduce cytosolic Ca^{2+} concentrations in airway smooth muscle cells. The concentration of magnesium is lower in serum and ERYTHROCYTES in asthmatic patients compared to normal controls and correlates with airway hyperresponsiveness [69], although the improvement in acute severe ASTHMA after magnesium does not correlate with plasma concentrations. More studies are needed before intravenous and inhaled magnesium sulfate are routinely recommended for the management of acute severe ASTHMA. There are too few studies in acute exacerbations of COPD to make any firm recommendation [70].

K^+ channel openers

Potassium (K^+) channels are involved in recovery of excitable cells after depolarization and therefore are important in stabilization of cells. K^+ channel openers such as cromakalim or levcromakalim (the *levo*-isomer of cromakalim) open ATP-dependent K^+ channels in smooth muscle and therefore relax airway smooth muscle [71]. This suggests that K^+ channel activators may be useful bronchodilators [72]. Clinical studies in ASTHMA have been disappointing with no bronchodilatation or protection against bronchoconstrictor challenges. However, the cardiovascular side effects of these drugs (postural hypotension, flushing) limit the oral dose. Inhaled K^+ also has problems, but new developments include K^+ channel openers that open calcium-activated large conductance K^+ channels (maxi-K channels) that are also opened by β_2-agonists, and these drugs may be better tolerated. Maxi-K channel openers also inhibit mucus secretion and cough, so may be of particular value in the treatment of COPD.

Atrial natriuretic peptides

Atrial natriuretic peptide (ANP) activates guanylyl cyclase and increases cGMP, leading to bronchodilatation. ANP and the related peptide urodilatin are bronchodilators in ASTHMA and give comparable effects to β_2-agonists [73, 74]. Since they work *via* a different mechanism from β_2-agonists they may give additional bronchodilatation that may be useful in acute severe ASTHMA when β_2-RECEPTOR function might be impaired.

VIP analogues

Vasoactive intestinal polypeptide (VIP) is a potent bronchodilator of human airways *in vitro*, but is not effective in patients as it is metabolized and also causes vasodilator side effects. More stable analogs of VIP, such as Ro 25-1533, which selectively stimulates VIP (VPAC2) RECEPTORS in airway smooth muscle, have been synthesized. Inhaled Ro 25-1533 has a rapid bronchodilator effect in asthmatic patients but is not as prolonged as formoterol [75].

Smooth muscle inhibitors

Drugs that inhibit the contractile machinery in airway smooth muscle, including rho kinase inhibitors, inhibitors of myosin light chain kinase and direct smooth muscle myosin inhibitors are also in development. As these agents also cause vasodilatation it will be necessary to administer them by inhalation.

Cromones

Sodium cromoglycate is a derivative of khellin, an Egyptian herbal remedy and was found to protect against allergen challenge without any bronchodilator effect. A structurally related drug, nedocromil sodium, which has similar pharmacological profile to cromoglycate, was subsequently developed. Although cromoglycate was popular in the past because of its good safety profile, its use has sharply declined with the more widespread use of the more effective inhaled corticosteroids, particularly in children.

Mode of action

Initial investigations indicated that cromoglycate inhibited the release of mediators by allergen in passively sensitized human and animal lung, and inhibited passive cutaneous ANAPHYLAXIS in rat, although it was without effect in guinea pig. This activity was attributed to stabilization of the mast cell membrane and thus cromoglycate was classified as a mast cell stabilizer. However, cromoglycate has a rather low potency in stabilizing human lung MAST CELLS, and other drugs which are more potent in this respect have little or no effect in clinical ASTHMA. This has raised doubts about mast cell stabilization as the mode of action of cromoglycate.

Cromoglycate and nedocromil potently inhibit bronchoconstriction induced by sulfur dioxide, metabisulfite and BRADYKININ, which are believed to act through activation of sensory nerves in the airways. In dogs, cromones suppress firing of unmyelinated C-fiber nerve endings, reinforcing the view that they might be acting to suppress sensory nerve activation and thus neurogenic INFLAMMATION. Cromones have variable inhibitory actions on other inflammatory cells that may participate in allergic INFLAMMATION, including MACROPHAGES and EOSINOPHILS. *In vivo* cromoglycate is capable of blocking the early response to allergen (which may be mediated by MAST CELLS) but also the late response and airway hyperresponsiveness, which are more likely to be mediated by other inflammatory cells. There is no convincing evidence that cromones reduce INFLAMMATION in asthmatic airways. The molecular mechanism of action of cromones is not understood, but some evidence suggests that they may block a particular type of chloride channel that may be expressed in sensory nerves, MAST CELLS and other inflammatory cells [76]. Their effects are mimicked in clinical challenge studies by furosemide, which also has an effect on chloride channel function (in addition to its diuretic action).

Current clinical use

Cromoglycate is a prophylactic treatment and needs to be given regularly. Cromoglycate protects against various indirect bronchoconstrictor stimuli, such as exercise, irritants and fog. It is only effective in mild ASTHMA, but is not effective in all patients, and there is no way to predicting which patients will respond. Cromoglycate was often used previously as the controller drug of choice in children because it lacks side effects. However, there is an increasing tendency to use low dose ICS instead as they are safe and far more effective. In adults, ICS are preferable to cromones, as they are effective in all patients. Cromoglycate has a very short duration of action and has to be given four times daily to provide good protection, which makes it much less useful than inhaled steroids that may be given once or twice daily. It may also be taken prior to exercise in children with exercise-induced ASTHMA that is not blocked by an inhaled β_2-agonist. In clinical practice, nedocromil has a similar EFFICACY to cromoglycate, but has the disadvantage of an unpleasant taste. Systematic reviews indicate that cromoglycate is largely ineffective in long-term control of ASTHMA in children and its use has now markedly declined [77]. The introduction of anti-LEUKOTRIENES has further eroded the market for cromones, as these drugs are of comparable or greater clinical EFFICACY and are more conveniently taken by mouth.

Side effects

Cromoglycate is one of the safest drugs available and side effects are extremely rare. The dry powder inhaler may cause throat irritation, coughing and, occasionally, wheezing but this is usually prevented by prior administration of a β_2-agonist inhaler. Very rarely a transient rash and urticaria are seen and a few cases of pulmonary eosinophilia have been reported, all of which are due to HYPERSENSITIVITY.

Mediator antagonists

Many inflammatory mediators have been implicated in ASTHMA and COPD (see also chapter A7), suggesting that inhibition of synthesis or RECEPTORS of these mediators may be beneficial [78, 79]. However, because these mediators have similar effects, specific inhibitors have usually been disappointing in both ASTHMA and COPD treatment.

Anti-histamines

HISTAMINE mimics many of the features of ASTHMA and is released from MAST CELLS in acute asthmatic responses, suggesting that ANTI-HISTAMINES may be useful in ASTHMA therapy. Many trials of HISTAMINE H_1 RECEPTOR antagonists have been conducted, but there is little evidence of any useful clinical benefit, as demonstrated by a meta-analysis [80]. Newer ANTI-HISTAMINES, including cetirizine and azelastine, have some beneficial effects, but this may be unrelated to their H_1 RECEPTOR antagonism. ANTI-HISTAMINES are effective in controlling rhinitis and this may help to improve overall ASTHMA control [81]. ANTI-HISTAMINES are not recommended in the routine management of ASTHMA.

Ketotifen is described as a prophylactic anti-ASTHMA compound. Its predominant effect is H_1 RECEPTOR antagonism, and it is this anti-histaminic effect that accounts for its sedative effect. Ketotifen has little or no effect in placebo-controlled trials in clinical ASTHMA on acute challenge, airway hyperresponsiveness or symptoms [82]. Ketotifen does not have a steroid-sparing effect in children maintained on ICS. It is claimed that ketotifen has disease-modifying effects if started early in ASTHMA in children and may even prevent the development of ASTHMA in atopic children, but carefully controlled studies are needed to assess the validity of these claims.

Anti-leukotrienes

There is considerable evidence that cysteinyl-LEUKOTRIENES (cys-LTs) are produced in ASTHMA and that

they have potent effects on airway function, inducing bronchoconstriction, airway hyperresponsiveness, plasma exudation, mucus secretion and possibly eosinophilic INFLAMMATION (Fig. 6) [83]. This suggested that blocking the LEUKOTRIENE pathways with LEUKOTRIENE modifiers may be useful in the treatment of ASTHMA, leading to the development of 5'-LIPOXYGENASE (5-LO) enzyme inhibitors (of which zileuton is the only drug marketed) and several antagonists of the cys-LT_1 RECEPTOR, including montelukast, zafirlukast and pranlukast.

Clinical studies

LEUKOTRIENE antagonists inhibit the bronchoconstrictor effect of inhaled LTD_4 in normal and asthmatic volunteers. They also inhibit bronchoconstriction induced by a variety of challenges, including allergen, exercise and cold air, with approximately 50% inhibition. With aspirin challenge, in aspirin-sensitive asthmatic patients, there is almost complete inhibition of the response [84]. Similar results have been obtained with the 5-LO inhibitor zileuton. This suggests that there may be no additional advantage in blocking LTB_4 in addition to cys-LTs, although this may not be the case in other inflammatory diseases, such as RHEUMATOID ARTHRITIS and inflammatory bowel disease. These drugs are active by oral administration and this may confer an important advantage in chronic treatment, particularly in relation to compliance.

Anti-LEUKOTRIENES have been intensively investigated in clinical studies [85]. In patients with mild to moderate ASTHMA there is a significant improve-

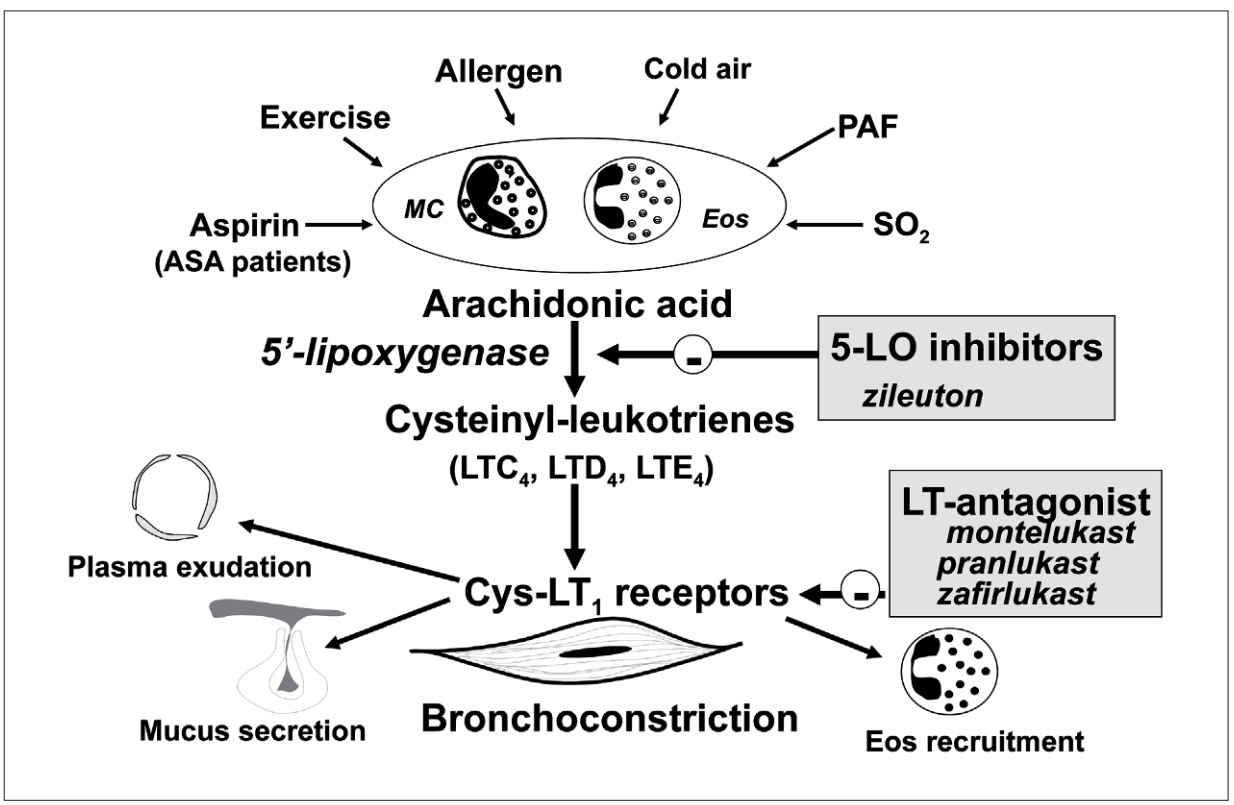

FIGURE 6. EFFECTS OF CYSTEINYL-LEUKOTRIENES ON THE AIRWAYS AND THEIR INHIBITION BY ANTI-LEUKOTRIENES
PAF, platelet-activating factor; LT, leukotriene; 5-LO, 5'-lipoxygenase; ASA, aspirin sensitive asthmatic

ment in lung function (clinical FEV_1 and home peak expiratory flow measurements) and ASTHMA symptoms, with a reduction in the use of rescue inhaled β_2-agonists. In several studies there is evidence for a bronchodilator effect, with an improvement in baseline lung function, suggesting that LEUKOTRIENES are contributing to the baseline bronchoconstriction in ASTHMA, although this varies between patients. However, anti-LEUKOTRIENES are considerably less effective than inhaled corticosteroids in the treatment of mild ASTHMA and cannot be considered as the treatment of first choice [86]. Anti-LEUKOTRIENES are therefore indicated more as an add-on therapy in patients not well controlled on inhaled corticosteroids. While they have add-on benefits, this effect is small and equivalent to doubling the dose of inhaled corticosteroid and less effective than adding an LABA [87]. In patients with severe ASTHMA who are not controlled on high doses of ICS and LABA, anti-LEUKOTRIENES do not appear to provide any additional benefit [88]. Theoretically, anti-LEUKOTRIENES should be of particular value in patients with aspirin-sensitive ASTHMA as they block the airway response to aspirin challenge, but, although anti-LEUKOTRIENES have some benefit in these patients, it is not any greater than in other types of ASTHMA [89]. Anti-LEUKOTRIENES are also effective in preventing exercise-induced ASTHMA, and are similar in EFFICACY to LABA in this respect [90]. Anti-LEUKOTRIENES also have a weak effect in rhinitis that may be additive to the effects of an ANTIHISTAMINE [91].

Studies have demonstrated weak anti-inflammatory effects of anti-LEUKOTRIENES in reducing EOSINOPHILS in sputum or in biopsies [92], but this is much less marked that with an ICS and there is no additional anti-inflammatory effect when added to an ICS [93]. Anti-LEUKOTRIENES, therefore, appear to act mainly as anti-bronchoconstrictor drugs, although they are clearly less effective in this respect than β_2-agonists, as they antagonize only one of several bronchoconstrictor mediators.

Cys-LTs have no role in COPD and are not elevated in exhaled breath condensate as in ASTHMA [79] and cys-LT_1 RECEPTOR antagonists have no role in therapy. By contrast, LTB_4, a potent neutrophil chemoattractant, is elevated in COPD, indicating that 5-LO inhibitors that inhibit LTB_4 synthesis may have some potential benefit by reducing neutrophil INFLAMMATION. However, a pilot study did not indicate any clear benefit of a 5-LO inhibitor in COPD patients [94].

Side effects

To date, anti-LEUKOTRIENES have been remarkably free of class-specific side effects. Zileuton causes an increased level of liver enzymes, so that monitoring of liver enzymes necessary with this drug, and high doses of zafirlukast may be associated with abnormal liver function. Montelukast is well tolerated in adults and children, with no significant adverse effects. The lack of side effects implies that LEUKOTRIENES do not appear to be important in any physiological functions. Several cases of Churg-Strauss syndrome have been associated with the use of zafirlukast and montelukast. Churg-Strauss syndrome is a very rare vasculitis that may affect the heart, peripheral nerves and kidney, and is associated with increased circulating EOSINOPHILS and ASTHMA. It is uncertain whether the cases reported so far are caused by a reduction in oral or inhaled corticosteroid dose rather than as a direct effect of the drug, although cases of Churg-Strauss syndrome have been described in patients on anti-LEUKOTRIENES who were not on concomitant corticosteroids therapy, suggesting that there is a causal link [95].

Future developments

One of the major advantages of anti-LEUKOTRIENES is that they are effective in tablet form. This may increase compliance with chronic therapy and makes treatment of children easier. Montelukast is effective as a once-daily preparation (10 mg in adults and 5 mg in children) and is therefore easy for patients to use. In addition, oral administration may treat concomitant allergic rhinitis. However, the currently available clinical studies indicate a relatively modest effect on lung function and symptom control, which is less for every clinical parameter measured than with ICS. This is not surprising, as there are many more mediators than cys-LTs involved in the pathophysiology of ASTHMA, and it is unlikely that antagonism of a single mediator could ever be

as effective as steroids, which inhibit all aspects of the inflammatory process in ASTHMA. Similarly, if anti-LEUKOTRIENES are functioning in ASTHMA as bronchodilators and anti-constrictors, it is unlikely that they will be as effective as a β_2-agonist, which will counteract bronchoconstriction, irrespective of the spasmogen. It is likely that anti-LEUKOTRIENES will be used less in the future as combination inhalers are used as the mainstay of ASTHMA therapy.

An interesting feature of the clinical studies is that some patients appear to show better responses than others, suggesting that LEUKOTRIENES may play a more important role in some patients. The variability in response to anti-LEUKOTRIENES may reflect differences in production of or responses to LEUKOTRIENES in different patients, and this in turn may be related to polymorphisms of 5-LO, LTC_4 synthase or cys-LT_1 RECEPTORS that are involved in the synthesis of LEUKOTRIENES [96].

It is unlikely that further advances can be made in cys-LT_1 RECEPTOR antagonists as montelukast is a once-daily drug that probably gives maximal RECEPTOR blockade. Cys-LT_2 RECEPTORS may be important in proliferative effects of cys-LTs in vascular and airway smooth muscle, and are not inhibited by current cys-LT_1 RECEPTOR antagonists [97]. The role of this RECEPTOR in ASTHMA is unknown so the value of also blocking cys-LT_2 is uncertain. 5-LO inhibitors have some potential in COPD, cystic fibrosis and possible severe ASTHMA as LTB_4 may contribute to neutrophil chemoattraction in the lungs of these conditions.

Immunomodulatory therapies

Immunosuppressive therapy has been considered in ASTHMA when other treatments have been unsuccessful or to reduce the dose of oral steroids required [98]. They are, therefore, only indicated in a very small proportion of asthmatic patients (<1%) at present. Most immunosuppressive treatments have a greater propensity for side effects than oral corticosteroids, and therefore cannot be routinely recommended. The role of immunosuppressive therapy in COPD and cystic fibrosis has not been evaluated.

Methotrexate

Low-dose METHOTREXATE (15 mg weekly) has a steroid-sparing effect in ASTHMA and may be indicated when oral steroids are contraindicated because of unacceptable side effects (e.g., in postmenopausal women when OSTEOPOROSIS is a problem). Some patients show better responses than others, but whether a patients will have a useful steroid-sparing effect is unpredictable. Overall, METHOTREXATE has a small steroid-sparing effect that is insufficient to significantly reduce side effects of systemic steroids, and this needs to be offset against the relatively high risk of side effects [99]. Side effects of METHOTREXATE are relatively common and include nausea (reduced if METHOTREXATE is given as a weekly injection), BLOOD DYSCRASIAS and hepatic damage. Careful monitoring of such patients (monthly blood counts and liver enzymes) is essential. Rarely pulmonary infections, pulmonary fibrosis and even death may occur. METHOTREXATE is disappointing in clinical practice and is now little used.

Gold

Gold has long been used in the treatment of chronic arthritis. A controlled trial of an oral gold preparation (Auranofin) demonstrated some steroid-sparing effect in chronic asthmatic patients maintained on oral steroids. Side effects such as skin rashes and nephropathy are a limiting factor. Overall gold provides little benefit in view of its small therapeutic ration and is not routinely used [100].

Ciclosporin

CICLOSPORIN (also know as cyclosporin A) is active against $CD4^+$ LYMPHOCYTES and therefore should be useful in ASTHMA, in which these cells are implicated. A trial of low-dose oral CICLOSPORIN in patients with steroid-dependent ASTHMA indicated that it can improve control of symptoms in patients with severe ASTHMA on oral steroids, but other trials have been unimpressive and, overall, its poor EFFICACY is outweighed by its side effects [101]. Side effects, such

as nephrotoxicity and hypertension, are common and there are concerns about long-term IMMUNOSUPPRESSION. In clinical practice, it has proved to be very disappointing as a steroid-sparing agent.

Intravenous immunoglobulin

Intravenous immunoglobulin (IVIG) has been reported to have steroid-sparing effects in severe steroid-dependent ASTHMA when high doses are used (2 g/kg), although a placebo-controlled double-blind trial in children showed that it is ineffective. IVIG reduces the production of IgE from B LYMPHOCYTES, and this may be the rationale for its use in severe asthmas. The relatively poor effectiveness and very high cost of this treatment mean that it is not generally recommended.

Specific immunotherapy

Theoretically SPECIFIC IMMUNOTHERAPY with common ALLERGENS should be effective in preventing ASTHMA. While this treatment is effective in allergic rhinitis due to single ALLERGENS (see chapter C5), there is little evidence that desensitizing injections to common ALLERGENS are very effective in controlling chronic ASTHMA [102]. Double-blind placebo-controlled studies have demonstrated poor effect in chronic ASTHMA in adults and children. Overall, the benefits of SPECIFIC IMMUNOTHERAPY are small in ASTHMA and there are no well-designed studies comparing this treatment to effective treatments such as ICS. Sublingual IMMUNOTHERAPY is safer, but its role in ASTHMA therapy has not yet been defined. Because there is a risk of anaphylactic and local reactions, and because the course of treatment is time consuming, this form of therapy cannot be routinely recommended for ASTHMA. The cellular mechanisms of SPECIFIC IMMUNOTHERAPY are of interest as this might lead to safer and more effective approaches in the future. SPECIFIC IMMUNOTHERAPY induces the secretion of the anti-inflammatory cytokine IL-10 from regulatory T helper LYMPHOCYTES (Treg) and this blocks costimulatory signal transduction in T cells (*via* CD28) so that they are unable to react to ALLERGENS presented by antigen-presenting cells [103]. In the future, more specific immunotherapies may be developed with cloned allergen EPITOPES, T cell peptide fragments of ALLERGENS, CpG oligonucleotides or Toll-like RECEPTOR (TLR)-9-allergen conjugate vaccines to stimulate Th1 immunity and suppress Th2 immunity [104].

Anti-IgE

Increased specific IgE is a fundamental feature of allergic ASTHMA (see also chapter A9). Omalizumab is a HUMANIZED monoclonal ANTIBODY that blocks the binding of IgE to high-AFFINITY IgE RECEPTORS (FcεR1) on MAST CELLS and thus prevents their activation by ALLERGENS (Fig. 7) [105]. In addition, it blocks binding on IgE to low-AFFINITY IgE RECEPTORS (FcεRII, CD23) on other inflammatory cells, including T and B LYMPHOCYTES, MACROPHAGES and possibly EOSINOPHILS to inhibit chronic INFLAMMATION. It also results in a reduction in circulating IgE levels. The ANTIBODY has a high AFFINITY and blocks IgE RECEPTORS by over 99%, which is necessary because of the AMPLIFICATION of these RECEPTORS.

Clinical use

Omalizumab is used for the treatment of patients with severe ASTHMA. The ANTIBODY is administered by subcutaneous injection every 2–4 weeks and the dose is determined by the titer of circulating IgE. Omalizumab reduces the requirement for oral and inhaled corticosteroids and markedly reduces ASTHMA exacerbations. It is also beneficial in treating allergic rhinitis. Because of its very high cost this treatment is likely to be used only in patients with very severe ASTHMA who are poorly controlled even on oral corticosteroids and in patients with very severe concomitant allergic rhinitis [106]. It may not be useful in concomitant atopic dermatitis due to the high levels of circulating IgE which cannot be neutralized. It may also be of value in protecting against ANAPHYLAXIS during SPECIFIC IMMUNOTHERAPY.

Side effects

Omalizumab is well tolerated and anaphylactic responses are very uncommon (<0.1%) [107].

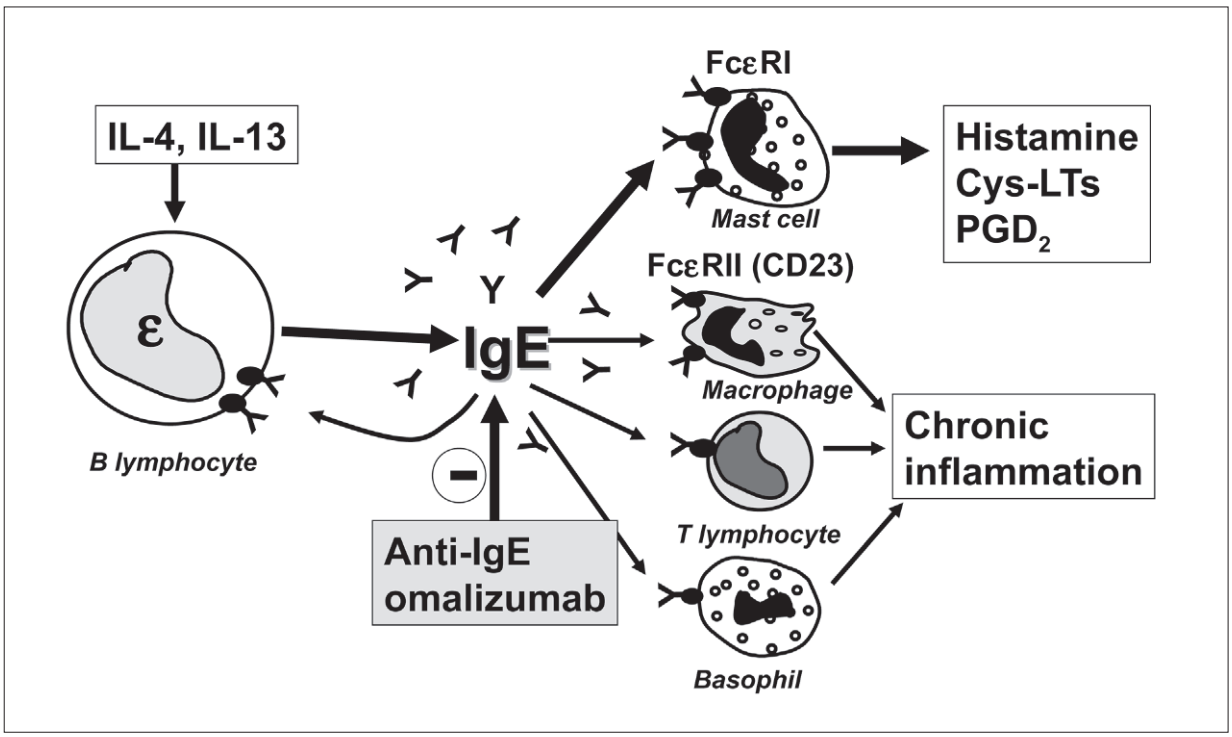

Figure 7

IgE plays a central role in allergic diseases and blocking IgE using an antibody, such as omalizumab, is a logical approach. IgE may activate high-affinity receptors (FcεRI) on mast cells as well as low-affinity receptors (FcεRII, CD23) on other inflammatory cells. IL, interleukin; cys-LT, cysteinyl-leukotriene; PG, prostaglandin

New drugs for asthma and COPD

Several new classes of drug are now in development for ASTHMA and COPD that are directed at the underlying chronic inflammatory process [108–110]. The INFLAMMATION in ASTHMA and COPD are different, so that different approaches are needed, but there are some common inflammatory mechanisms. Indeed, patients with severe ASTHMA have an inflammatory process that becomes more similar to that in COPD, suggesting that drugs that are effective in COPD may also be useful in patients with severe ASTHMA that is not well controlled with corticosteroids [2]. In ASTHMA many new therapies have targeted eosinophilic INFLAMMATION. In COPD a better understanding of the inflammatory process has highlighted several new therapeutic targets.

Need for new treatments

Asthma

Current ASTHMA therapy is highly effective and the majority of patients can be well controlled with ICS and short- and long-acting β_2-agonists, particularly combination inhalers. These treatments are not only effective, but safe and relatively inexpensive. This poses a challenge for the development of new treatments, since they will need to be safer, at least as effective as existing treatments, or offer some other advantage in long-term disease management. However, there are problems with existing therapies:

- Existing therapies have side effects as they are not specific for ASTHMA. Inhaled β_2-agonists may

have side effects and there is some evidence for the development of TOLERANCE, especially to their bronchoprotective effects. ICS also may have local and systemic side effect at high doses and there is still a fear of using long-term steroid treatment in many patients. Other treatments, such as theophylline, anticholinergics and anti-LEUKOTRIENES are less effective and are largely used as add-on therapies.

- There is still a major problem with poor compliance (ADHERENCE) in the long-term management of ASTHMA, particularly as symptoms come under control with effective therapies [111]. It is likely that a once-daily tablet or even an infrequent injection may give improved compliance. However, oral therapy is associated with a much greater risk of systemic side effects and therefore needs to be specific for the abnormality in ASTHMA.
- Patients with severe ASTHMA (approximately 5–10% of total) are often not controlled on maximal doses of inhaled therapies or may have serious side effects from therapy, especially oral corticosteroids. These patients are relatively resistant to the anti-inflammatory actions of corticosteroids and require some other class of therapy to control the asthmatic process.
- None of the existing treatments for ASTHMA is disease modifying, which means that the disease recurs as soon as treatment is discontinued.
- None of the existing treatments is curative, although it is possible that therapies which prevent the immune aberration of ALLERGY may have the prospects for a cure in the future.

COPD

In sharp contrast to ASTHMA, there are few effective therapies in COPD, despite the fact that it is a common disease that is increasing worldwide [112].

- The neglect of COPD is probably a result of several factors:
- COPD is regarded as largely irreversible and is treated as poorly responsive ASTHMA.
- COPD is self-inflicted and therefore does not deserve investment.
- There are few satisfactory animal models that closely mimic the human disease.
- Relatively little is understood about the cell and molecular biology of this disease or even about the relative roles of small airways disease and parenchymal destruction.

None of the treatments currently available prevent the progression of the disease, and yet the disease is associated with an active inflammatory process that results in progressive obstruction of small airways and destruction of lung parenchyma. Increased understanding of COPD will identify novel targets for future therapy [110] (Fig. 8).

Development of new therapies

Several strategies have been adopted in the search for new therapies:

- Improvement of existing classes of drug: This is well exemplified by the increased duration of β_2-agonists with salmeterol and formoterol and of anticholinergics with tiotropium bromide, and with the improved pharmacokinetic of the inhaled corticosteroids budesonide, fluticasone propionate, mometasone and ciclesonide, with increased first-pass metabolism and therefore reduced systemic absorption.
- Development of novel therapies through better understanding of the disease process. Examples are anti-IL-5 as a potential treatment of ASTHMA and PDE4 inhibitors as an anti-inflammatory therapy for COPD.
- Serendipitous observations, often made in other therapeutic areas. Examples are anti-TNF therapies for airway diseases, derived from observations in other chronic inflammatory diseases.
- Identification of novel targets through gene and protein profiling. This approach will be increasingly used to identify the abnormal expression of genes (molecular genomics) and proteins (PROTEOMICS) from diseased cells, and through identification of single nucleotide polymorphisms (SNPs) that contribute to the disease process. So far this approach has not led to the development of any useful therapies.

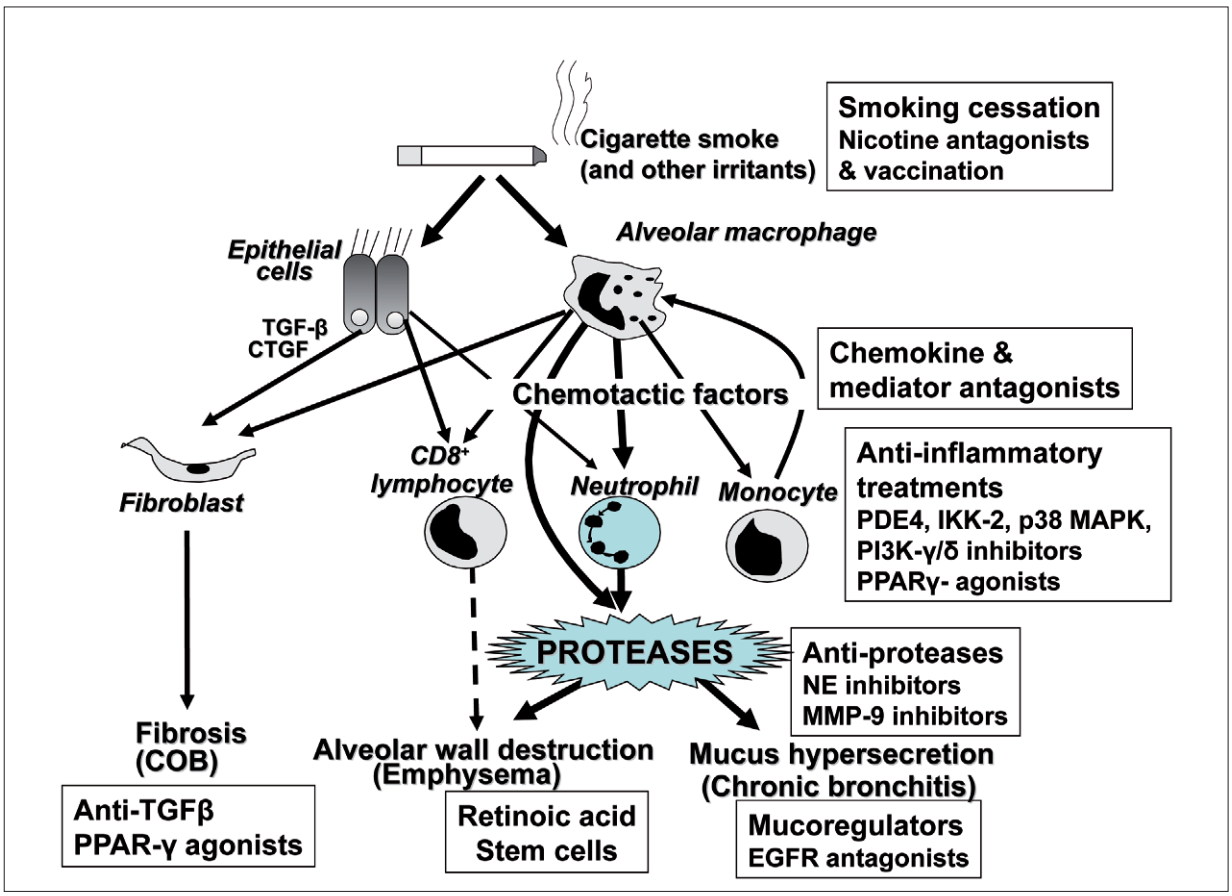

FIGURE 8.

Cigarette smoke (and other irritants) activates macrophages in the respiratory tract that release multiple chemotactic factors that attract neutrophils, monocytes and T lymphocytes (particularly $CD8^+$ cells). Several cells also release proteases, such as neutrophil elastase (NE) and matrix metalloproteinase-9 (MMP-9), which the break down connective tissue in the lung parenchyma (emphysema) and also stimulate mucus hypersecretion (chronic bronchitis). $CD8^+$ may also be involved in alveolar wall destruction. Transforming growth factor (TGF)-β and connective tissue growth factor (CTGF) released from inflammatory cells may mediate small airway fibrosis. The inflammatory process may be inhibited at several stages (shown in the boxes).

PDE, phosphodiesterase; IKK, inhibitor of nuclear factor-κB kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3-kinase; PPAR, peroxisome proliferator activated receptor; COB, chronic obstructive bronchitis; CB, cannabinoid; EGFR, epithelial growth factor receptor

Novel mediator antagonists

Blocking the RECEPTORS or synthesis of inflammatory mediators is a logical approach to the development of new treatments for ASTHMA and COPD. However, in both diseases many different mediators are involved and therefore blocking a single mediator is unlikely to be very effective, unless it plays a key role in the disease process [78, 79]. Several specific mediator antagonists have been found to be ineffective in

ASTHMA, including antagonists/inhibitors of THROMBOXANE, PLATELET-ACTIVATING FACTOR, BRADYKININ and tachykinins. However, these blockers have often not been tested in COPD, in which different mediators are involved.

CRTh2 antagonists

A chemotactic factor for Th2 cells has been identified as PROSTAGLANDIN D_2, which acts on a DP_2 receoptor. Several CRTh2 antagonists are now in development for ASTHMA with promising initial results [113].

Endothelin antagonists

ENDOTHELIN has been implicated in some of the structural changes that occur in ASTHMA and COPD. ENDOTHELIN antagonists are approved for the treatment of pulmonary hypertension (see below) and might be useful in treating the structural changes that occur in ASTHMA and COPD, but so far have not been tested.

Antioxidants

Oxidative stress is important in severe ASTHMA and COPD and may contribute to corticosteroid resistance. Existing antioxidants include vitamins C and E and *N*-acetyl-cysteine, but these drugs have weak effects; however, more potent antioxidants are in development [114].

Inducible NO synthase inhibitors

NITRIC OXIDE (NO) production is increased in ASTHMA and COPD as a result of increased inducible NO synthase (iNOS) expression in the airways. NO and oxidative stress generate PEROXYNITRITE, which may nitrate proteins, leading to altered cell function. Several selective iNOS inhibitors are now in development [115] and one of these, L-N^6-(1-imminoethyl) lysine (L-NIL), gives a profound and long-lasting reduction in the concentrations of NO in exhaled breath [116]. An iNOS inhibitor was found to be ineffective in ASTHMA, however [117].

Cytokine modifiers

CYTOKINES play a critical role in perpetuating and amplifying the INFLAMMATION in ASTHMA and COPD, suggesting that anti-CYTOKINES may be beneficial as therapies [118]. Although most attention has focused on inhibition of CYTOKINES, some CYTOKINES are anti-inflammatory and might have therapeutic potential.

IL-5 plays a pivotal role in eosinophilic INFLAMMATION and is also involved in eosinophil survival and priming. It is an attractive TARGET in ASTHMA, as it is essential for eosinophilic INFLAMMATION, there do not appear to be any other CYTOKINES with a similar role, and lack of IL-5 in gene knockout mice does not have any deleterious effect. Blocking anti-IL-5 ANTIBODIES inhibit eosinophilic INFLAMMATION and airway hyperresponsiveness in animal models of ASTHMA, and markedly reduce circulating and airway EOSINOPHILS in ASTHMA patients, but have no effect on airway hyperresponsiveness or clinical features of ASTHMA [119, 120]. In highly selected patients with severe ASTHMA and persistent eosinophilia, despite high does of corticosteroids, there is a significant reduction in exacerbations [121]. Blocking IL-4 and IL-13, which determine IgE synthesis, has so far proved to be ineffective in clinical studies.

TNF-α may play a key role in amplifying airway INFLAMMATION, through the activation of NF-κB, AP-1 and other transcription factors. TNF-α production is increased in ASTHMA and COPD, and in the latter may be associated with the cachexia and weight loss that occurs in some patients with severe COPD. Anti-TNF-α blocking ANTIBODIES were shown to be ineffective in COPD patients and in patients with severe ASTHMA, while apparently increasing infections and malignancies [122, 123].

Chemokine receptor antagonists

Many CHEMOKINES are involved in ASTHMA and COPD and play a key role in recruitment of inflammatory cells, such as EOSINOPHILS, NEUTROPHILS, MACROPHAGES and LYMPHOCYTES into the lungs. Chemokine RECEPTORS are attractive targets, as they are seven-transmembrane spanning proteins, like adrenergic RECEPTORS, and small molecule inhibitors are now in development [124, 125]. In ASTHMA, CCR3 antagonists,

which should block eosinophil recruitment into the airways, are the most favored TARGET, but several small molecule CCR3 antagonists have failed because of toxicology problems. In COPD, CXCR2 antagonists, which prevent neutrophil and monocyte CHEMOTAXIS due to CXC CHEMOKINES such as CXCL1 and CXCL8, have been effective in animal models of COPD and in neutrophilic INFLAMMATION in normal subjects, and are now in clinical trials in COPD patients.

Protease inhibitors

Several proteolytic enzymes are involved in the chronic INFLAMMATION of airway diseases. Mast cell tryptase has several effects on airways, including increasing responsiveness of airway smooth muscle to constrictors, increasing plasma exudation, potentiating eosinophil recruitment and stimulating fibroblast proliferation. Some of these effects are mediated by activation of the proteinase-activated RECEPTOR PAR2. Tryptase inhibitors have so far proved to be disappointing in clinical studies.

Several proteases are involved in the degradation of connective tissue in COPD, particularly enzymes that break down elastin fibers, such as neutrophil elastase and matrix METALLOPROTEINASES (MMP), which are involved in emphysema. Neutrophil elastase inhibitors have been difficult to develop and there are no positive clinical studies in COPD patients. MMP9 appears to be the predominant elastolytic enzyme in emphysema and several selective inhibitors are now in development.

New anti-inflammatory drugs

ICS are by far the most effective therapy for ASTHMA, yet are ineffective in COPD and poorly effective in severe ASTHMA. This has led to the search for alternative broad-spectrum anti-inflammatory treatments for ASTHMA and COPD (Tab. 6).

Table 6. New anti-inflammatory drugs for asthma and COPD

New glucocorticoids (ciclesonide, dissociated steroids)
Immunomodulators (inhaled ciclosporin, tacrolimus, rapamycin, mycophenolate mofetil)
Phosphodiesterase-4 inhibitors (cilomilast, roflumilast)
p38 MAP kinase inhibitors
NF-κB inhibitors (IKK-2 inhibitors)
Adhesion molecule blockers (VLA4 antibody, selectin inhibitors)
Cytokine inhibitors (anti-IL-4, anti-IL-5, anti-IL-13, anti-TNF antibodies)
Anti-inflammatory cytokines (IL-1ra, IFN-γ, IL-10, IL-12)
Chemokine receptor antagonists (CCR3, CCR2, CXCR2 antagonists)
Peptides for immunotherapy
Vaccines

Phosphodiesterase inhibitors

PDEs break down cyclic nucleotides that inhibit cell activation and at least 11 families of enzymes have been characterized. Theophylline, long used as an ASTHMA treatment, is a weak but non-selective PDE inhibitor. PDE4 is the predominant family of PDEs in inflammatory cells, including MAST CELLS, EOSINOPHILS, NEUTROPHILS, T LYMPHOCYTES, MACROPHAGES and structural cells such as sensory nerves and epithelial cells [126]. This suggests that PDE4 inhibitors could be useful as an anti-inflammatory treatment in both ASTHMA and COPD (Fig. 9). In animal models of ASTHMA, PDE4 inhibitors reduce eosinophil infiltration and airway hyperresponsiveness to allergen, whereas in COPD they are effective against smoke-induced INFLAMMATION and emphysema. Several PDE4 inhibitors have been tested clinically, but with disappointing results, as the dose has been limited by side effects, particularly nausea, vomiting, headaches and diarrhea. In COPD, an oral PDE4 inhibitor roflumilast has some effect in reducing exacerbations and improving lung function, but the dose is limited by side effects [127]. There are four subfamilies of PDE4, and it now seems that PDE4D is the major enzyme

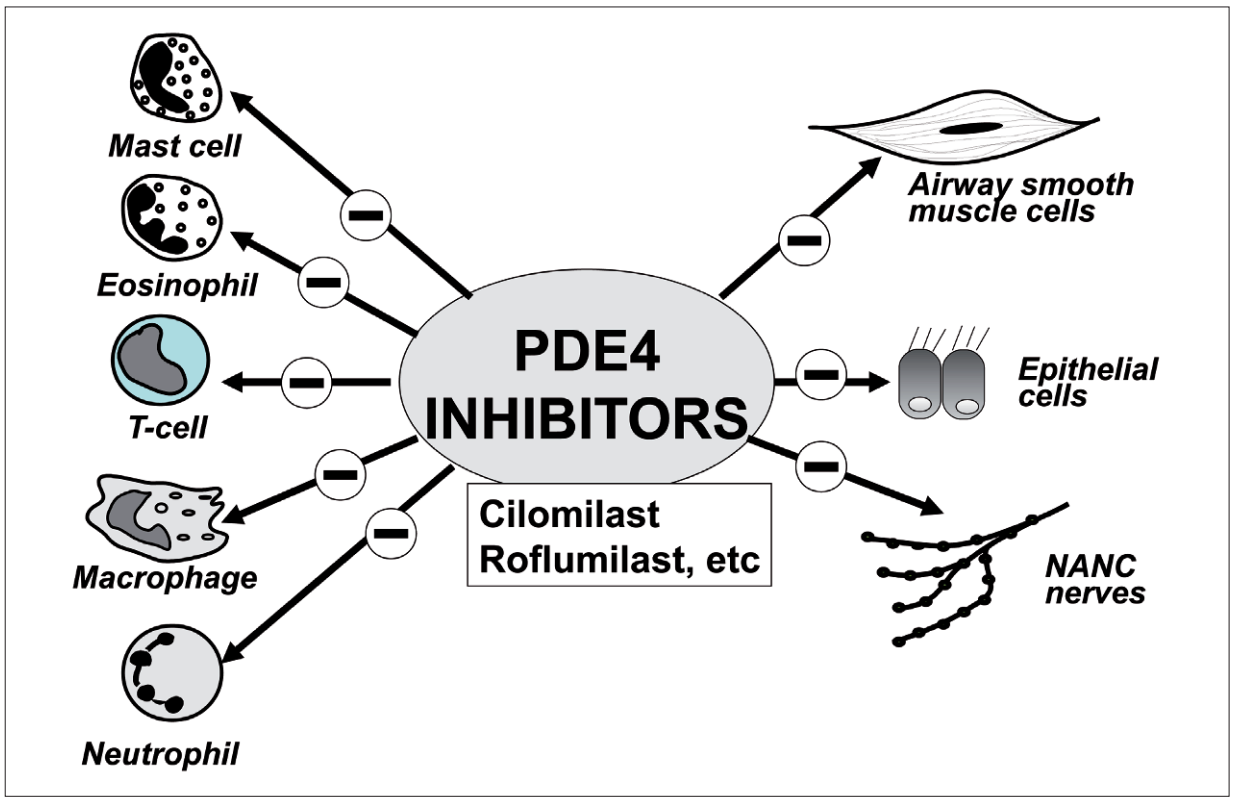

Figure 9.
Inhibitors of phosphodiesterase 4 (PDE4) may be useful anti-inflammatory treatments in COPD and asthma as they inhibit several aspects of the inflammatory process.

mediating vomiting, whereas PDE4B is important for anti-inflammatory effects. This suggests that selective PDE4B inhibitors might have a greater therapeutic ratio. Another approach has been to develop inhaled PDE4 inhibitors, but this has so far proved to be ineffective. PDE5 inhibitors are vasodilators that are used in the treatment of pulmonary hypertension (see below).

NF-κB inhibitors

NF-κB plays an important role in the orchestration of chronic INFLAMMATION and many of the inflammatory genes that are expressed in ASTHMA and COPD are regulated by this transcription factor [128]. This has prompted a search for specific blockers of these transcription factors. NF-κB is naturally inhibited by the inhibitory protein IκB, which is degraded after activation by specific kinases. Small molecule inhibitors of the IκB kinase IKK2 (or IKKβ) are in clinical development [129, 130]. These drugs may be of particular value in COPD where corticosteroids are largely ineffective. However, there are concerns that inhibition of NF-κB may cause side effects such as increased susceptibility to infections, which as been observed in gene disruption studies when components of NF-κB are inhibited.

MAP kinase inhibitors

There are three major MITOGEN-activated protein (MAP) kinase pathways, and there is increasing rec-

ognition that these pathways are involved in chronic INFLAMMATION [131]. There has been particular interest in the p38 MAP kinase pathway that is blocked by a novel class of drugs, such as SB203580 and RWJ67657. These drugs inhibit the synthesis of many INFLAMMATORY CYTOKINES, CHEMOKINES and inflammatory enzymes [132]. p38 MAPK inhibitors are in development for the treatment of ASTHMA as they inhibit Th2 cytokine synthesis and for COPD as they inhibit neutrophilic INFLAMMATION and INFLAMMATORY CYTOKINES and CHEMOKINES. However, clinical studies have revealed marked adverse effects and toxicities, so that inhaled delivery is being explored.

Summary

Pharmacological therapies are the mainstay of management of ASTHMA and COPD. Current bronchodilators relax airway smooth muscle and include β_2-adrenergic agonists, muscarinic RECEPTOR antagonists and theophylline. Long-acting inhaled β_2-agonists are an important advance, and these are very effective add-on therapy to controller drugs. Although new classes of bronchodilator, such as potassium channel openers and vasoactive intestinal peptide analogs, have been explored, they are unlikely to be as effective as β_2-agonists, which act as functional antagonists to counteract all bronchoconstrictor mechanisms. Controller drugs act on the underlying disease by suppressing the inflammatory process. Cromones are much less effective in ASTHMA and are now little used. Theophylline may also have anti-inflammatory effects in low doses and is a useful add-on therapy in more severe ASTHMA. Many mediators are involved in ASTHMA and COPD, so blocking a single mediator is unlikely to have a major beneficial effect. Anti-LEUKOTRIENES have relatively weak effects in ASTHMA compared to corticosteroids. Steroid-sparing therapies, such as METHOTREXATE and CICLOSPORIN, are only indicated in patients who have side effects from maintenance oral steroids, but these drugs often have worse side effects so are little used. Anti-IgE has recently been introduced for the treatment of very severe ASTHMA. There are several new classes of controller drugs now in development for ASTHMA and COPD, including PDE4 inhibitors and chemokine RECEPTOR antagonists.

Selected reading

Barnes PJ, Drazen JM, Rennard SI, Thomson NC. *Asthma and COPD*, Amsterdam: Elsevier 2009; pp 1–878

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http://www.ginasthma.com/
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Perspectives of immunotherapy in the management of asthma and other allergic conditions

Anthony J. Frew

Introduction

ASTHMA treatment is now very structured and organised as a result of the introduction of treatment guidelines, which have brought specialist approaches to the care of ASTHMA patients throughout the medical community. It is now generally accepted that, at the population level, we have reached a plateau in terms of the level of control that can be achieved with standard drugs. This is enough for most patients to lead relatively normal lives with a low chance of serious exacerbations. At the individual patient level, there is still room to improve compliance and control, but we cannot expect to abolish all ASTHMA symptoms simply by adjusting the doses and delivery devices used for standard drugs. We accept that our current therapies, such as inhaled steroids, do not abolish the disease, but merely control the symptoms. Moreover, it is unclear whether their long-term use prevents or diminishes the risk of ASTHMA complications, such as the development of fixed airways obstruction. Thus, there is still scope for the development and use of new therapies to control and/or suppress ASTHMA.

A great deal is now known about the inflammatory and immunological basis of ASTHMA. Over the past 30 years, many different cells and molecules have been implicated in various aspects of the pathogenesis of ASTHMA [1]. When these various "key players" and "key mediators" were identified, we waited eagerly for the development of specific and effective inhibitors that would allow us to assess whether these cells and mediators are really important in the process of ASTHMA, or just coincidental bystanders.

General aspects of immunotherapy in allergy and asthma

In the context of ALLERGY and ASTHMA, the term "IMMUNOTHERAPY" generally refers to specific desensitising therapy, targeted against individual ALLERGENS, although increasingly this term is used in a broader sense to describe a range of immunological therapies that may influence allergic disease. Allergists and immunopharmacologists need to remember that the term "IMMUNOTHERAPY" is also used by oncologists and rheumatologists to describe quite different immunomodulatory treatments. It may therefore be more appropriate to define IMMUNOTHERAPY as any form of treatment that seeks to modify the immune response to improve or alleviate immunological diseases. Within this general framework, one can identify (a) non-specific treatments that are used to boost or suppress general immune functions, and (b) specific therapies that aim to modify the immune response to specific external or internal ANTIGENS, without having any effect on the response of the IMMUNE SYSTEM to unrelated ANTIGENS.

Fundamental to any appraisal of IMMUNOTHERAPY is a coherent understanding of the immunological basis of the particular disease being studied. Thus, in attempting to manipulate the IMMUNE SYSTEM in allergic diseases such as ASTHMA, it is crucial to establish which components of the disease have an immunological basis, as opposed to an inflammatory basis, and which of these immunological processes are necessary for the disease, but largely irreversible. For example, it is clear that an exaggerated tendency to develop IgE sensitisation is a substantial risk factor for the onset of ASTHMA, but much less clear whether

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allergic responses play a significant role in the maintenance of established ASTHMA. It is thus entirely possible that a particular immunotherapeutic approach could be effective in preventing the onset of disease, but have no impact on the established condition. Conversely, in patients in whom allergic reactions are relevant to the ongoing disease, specific or non-SPECIFIC IMMUNOTHERAPY may be a useful way of reducing the effect of allergic trigger factors and may hence alleviate clinical symptoms.

A central thesis of IMMUNOTHERAPY is the plasticity of the immune response in man. For most of the past 25 years our thinking has been dominated by the Th1/Th2 concept. This is based on a view that T helper cells regulate the immune and inflammatory response, through the production of immunomodulatory CYTOKINES, and that once the T cell has differentiated its cytokine pattern is stable. Most of the data showing that T cells acquire and retain cytokine profiles was generated *in vitro* using mouse cells: similar general patterns are evident in human cells, but there are clear inter-species differences. Our thinking is now moving away from a simple Th1-Th2 spectrum towards a concept in which the ANTIGEN-PRESENTING CELL plays a major role in determining the initial cytokine response of the T cell. We now believe that T cells are predisposed towards a particular phenotype, but whether they develop and proliferate appears to be regulated by specific T regulatory cells, which use CYTOKINES such as IL-10 and TGF-β to control T cell activity and function (see chapter A2). Nonetheless, it is unclear at which point in this process the T cell becomes irreversibly committed to a particular phenotype. This means we are now less certain whether driving the response from Th2 to Th1 *in vivo* involves changing the phenotype of committed Th2 cells, or the induction of previously uncommitted cells, which then acquire the Th1 phenotype.

Non-specific immunomodulatory therapies

Previous attempts to modulate the IMMUNE SYSTEM in ASTHMA have drawn on a range of immunosuppressive therapies that have been used in other disease areas. At one level, glucocorticosteroids are immunomodulatory: there is no doubt that they are effective agents in the control of ASTHMA in most patients, and they have complex effects on airways biology as well as on the inflammatory processes of ASTHMA (see chapters C4 and C15). Other general immunosuppressive agents studied in ASTHMA include CICLOSPORIN A, METHOTREXATE, AZATHIOPRINE, gold, troleandomycin, hydroxychloroquine, and intravenous immunoglobulin. In general, these agents have been used mainly for their steroid-sparing properties, rather than for any particular aspect of their pharmacology.

Specific mediator antagonists

A variety of newer treatments have been designed to TARGET specific components of the inflammatory process found in asthmatic airways (and are dealt with in more detail in chapter C4). These targets include IgE ANTIBODIES, specific CYTOKINES, CHEMOKINES and vascular ADHESION MOLECULES.

Anti-IgE

It is well established that IgE ANTIBODIES trigger release of inflammatory mediators from MAST CELLS, *via* attachment to high-AFFINITY IgE RECEPTORS and subsequent cross-linking (see chapter A3 and C2). IgE also plays a role in antigen presentation and in the development of T and B LYMPHOCYTES.

The serum concentration of free IgE is significantly reduced by treatment with humanised monoclonal anti-IgE ANTIBODIES, as is the response to inhaled antigen [2, 3]. Initial clinical trials suggest that anti-IgE treatment may allow significant reduction of glucocorticosteroid dose [4]. It has also been suggested that anti-IgE may affect the way that ANTIGENS are captured and presented to LYMPHOCYTES, by disrupting the IgE-focussed presentation of ANTIGENS by DENDRITIC CELLS [5]. It remains to be proven whether this is a biologically important effect of anti-IgE. Treatment with anti-IgE (omalizumab) improves symptom scores and lung function in patients with

moderate to severe ASTHMA [6,7], and reduces serum concentrations of IL-5 and IL-8, compared to pre-treatment values [8]. Anti-IgE therapy also reduces the expression of the high-AFFINITY IgE RECEPTOR on MAST CELLS in the human asthmatic airway [9]. Clinical trials have confirmed that anti-IgE is effective in patients with more severe forms of ASTHMA that are not controlled on high-dose inhaled steroids and long-acting β-agonists [10]. Because of cost issues, anti-IgE is mainly used as an add-on therapy in patients receiving higher doses of inhaled steroids, those who require frequent emergency treatment, and those with significantly impaired lung function [11].

Anti-cytokine therapy

Several CYTOKINES have been implicated in the development and expression of allergic ASTHMA. In particular, IL-4 plays a critical role in regulating B cell switching to make IgE, but also modulates mucus production and endothelial ADHESION MOLECULE expression [12]. IL-5 has a key role [13] in the development, recruitment and activation of EOSINOPHILS and some have suggested it may be more relevant to ASTHMA, whereas IL-4 may be the cytokine relevant to ALLERGY. IL-13 can substitute for IL-4 in B cell switching, and both IL-13 and tumour necrosis factor (TNF) have also been implicated in AIRWAYS REMODELLING [14–17].

Anti-IL-5 ANTIBODIES are very effective at blocking the development of eosinophilia and allergen-induced hyperresponsiveness in animal models [18–20]. In a clinical trial of monoclonal anti-IL-5 ANTIBODY (mepolizumab), the sputum eosinophil response to allergen inhalation was reduced, but allergen-induced early and late-phase responses were not affected [21]. This study was not really powered to address the clinical EFFICACY of anti-IL-5, but it has been widely cited as evidence that targeting IL-5 will not be an effective way of treating ASTHMA. Subsequent work has shown that anti-IL-5 is more effective at reducing sputum and blood eosinophilia than reducing tissue eosinophil numbers [22, 23]. An alternative anti-IL-5 ANTIBODY (SCH55700) has also undergone initial trials in patients with severe ASTHMA [24] and there has been a recent revival of interest in anti-IL-5 strategies, following the recognition that EOSINOPHILS may persist in the tissues after anti-IL-5, even if they are completely eliminated from the circulation [25].

Interleukin-4 (IL-4) affects IgE CLASS SWITCHING and endothelial ADHESION MOLECULE expression [26]. Inhalation of IL-4 causes increased sputum eosinophilia and increased bronchial responsiveness in patients with ASTHMA [27]. Inhibition of IL-4 with nebulised SYNTHETIC soluble IL-4 RECEPTOR (sIL-4R) molecules blocked eosinophil recruitment and mucus production in animal models of ASTHMA. An initial clinical trial was promising [28], but later studies proved less convincing. A similar approach has been proposed to TARGET IL-13 or the common chain of the IL-4 and IL-13 RECEPTOR with a number of candidate molecules currently undergoing clinical trials [29, 30].

TNF-α has been implicated in fibrosis and other aspects of AIRWAYS REMODELLING, It may therefore be important in the chronicity of ASTHMA, as distinct from the initial phases of allergic sensitisation, where IL-4 and IgE are more involved. An open study of a TNF-RECEPTOR antagonist has showed promise [31], and further, double-blind, placebo-controlled studies are now indicated [32].

Cytokine therapy

Several CYTOKINES have "anti-allergic" properties, either by promoting the Th1 pattern of response, or by opposing Th2 development. In particular, IFN-γ opposes the actions of IL-4, while IL-12 works upstream, influencing antigen-presenting cells so that they are biased towards the Th1 phenotype [33]. Parenteral administration of IFN-γ to steroid-dependent asthmatic patients caused a reduction in blood eosinophil numbers, but had no effect on lung function or oral steroid requirements [34]. The pro-inflammatory cytokine IL-12 helps to regulate the balance between Th1 and Th2 LYMPHOCYTES and biases the IMMUNE SYSTEM away from a Th2 response. Patients with allergic ASTHMA have reduced blood concentrations of IL-12 and their alveolar MACROPHAGES are deficient in IL-12 production [35]. Polymor-

phisms have been identified in the IL-12 promoter region, which are associated with ASTHMA and IgE levels. In animal models, exogenous IL-12 inhibits bronchial hyperresponsiveness and airway eosinophilia [36]. In mild ASTHMA, administration of IL-12 resulted in a reduction in blood eosinophil count and in sputum eosinophil count following allergen challenge. However, airway hyperresponsiveness and the late asthmatic reaction were not affected. Further development seems unlikely as IL-12 administration was associated with significant side effects in about a quarter of patients [37]. Concern about the potential pro-inflammatory properties of IL-12 and IFN-γ may limit their potential use as immunomodulators in ASTHMA. Moreover, IL-12 had little marginal benefit when used as a supplement to sublingual IMMUNOTHERAPY for house dust mite ALLERGY in children [38].

Anti-CD4 monoclonal antibody therapy

Targeting all T cells rather than individual CYTOKINES might eliminate those T cells currently responding to ANTIGENS, without damaging the T cells that are needed for defence against future encounters with pathogens. This anti-T cell strategy has been used successfully in RHEUMATOID ARTHRITIS and has been investigated in severe corticosteroid-dependent ASTHMA [39]. Significant improvements were observed in morning and evening PEFR and in symptom scores, in a randomised double-blind placebo controlled trial involving 22 patients. No serious adverse effects were seen.

T cell-specific immunosuppressive drugs

Several other new T cell-specific drugs may theoretically be useful in the treatment of ASTHMA, although clinical trials have not yet been reported. These include TACROLIMUS, rapamycin, mycophenolate mofetil and deoxysperguanolin (see also chapter C13). These have multiple actions including the inhibition of cytokine production and IgE-dependent HISTAMINE release, the blocking of T cell stimulation *via* the CD28 pathway and of T cell differentiation and proliferation [40].

Specific immunotherapy

Specific allergen IMMUNOTHERAPY (SIT) involves the administration of allergen extracts to modify or abolish symptoms associated with atopic ALLERGY. The treatment is targeted at those ALLERGENS recognised by the patient and physician as responsible for symptoms. A decision to use SPECIFIC IMMUNOTHERAPY therefore demands a careful assessment of the patient's condition and the role of allergic triggers. In addition, since the course of treatment is lengthy and relatively expensive, there must also be an assessment of the risks and costs as compared to symptomatic treatment with corticosteroids and bronchodilators.

IMMUNOTHERAPY was first developed in the late 19th century [41] and further developed in the US during the 1920s and 1930s. Many of the basic principles described then remain valid today. Usually, patients receive a course of injections, starting with a very low dose of allergen, and building up gradually until a plateau or maintenance dose is achieved. Maintenance injections are then given at 4–6-weekly intervals for 3–5 years (Tab. 1). The updosing phase is normally given as a series of weekly injections, but several alternative induction regimes have been tried, some giving the whole series of incremental injections in a single day (rush protocol), while others give the doses in clusters of several doses on one day then waiting a week before giving the next cluster of injections (semi-rush protocol). The main drawback to these rapid updosing regimes is the increased risk of adverse reactions compared to conventional protocols. On the other hand, full protection can be attained in a few days as compared to the 3 months required in the conventional regime. This is useful in patients being treated for life-threatening conditions such as ANAPHYLAXIS induced by hymenoptera (bee and wasp) stings, but less relevant to ASTHMA.

Mechanisms of immunotherapy

Several mechanisms have been proposed to explain the beneficial effects of IMMUNOTHERAPY. These include induction of IgG (blocking) ANTIBODIES, long-

Table 1. Injection schedule for grass pollen immunotherapy

Week	Injection	Allergen concentration	Dose (mL) injected	Amount of allergen injected (SQ-U)
1	1	1000	0.1	100
1	2	10000	0.1	1000
2	3	10000	0.2	2000
2	4	10000	0.4	4000
3	5	10000	0.6	6000
3	6	100000	0.1	10000
4	7	100000	0.1	10000
4	8	100000	0.2	20000
5	9	100000	0.3	30000
5	10	100000	0.3	30000
6	11	100000	0.5	50000
6	12	100000	0.5	50000

Subsequently, maintenance injections (100000 SQ-U) are given every 4–6 weeks for a total of 3 years

term reductions in specific IgE, reduced recruitment of effector cells, altered T cell cytokine balance, induction of T cell anergy and the induction of regulatory T cells. Whether given by injection or sublingually, specific immunotherapy induces changes in T cell and antibody responses. The challenge for clinical scientists has been to work out which of the observed changes drive the clinical benefit, and which are just epiphenomena. Allergen-specific IgE levels rise temporarily during the initial phase of specific immunotherapy, but fall back to pre-treatment levels during maintenance therapy [42]. The immediate weal and flare response to skin testing usually declines during the initial phases of specific immunotherapy but this effect is relatively small compared to the degree of clinical benefit. In contrast, the late-phase response to skin testing is virtually abolished after successful specific immunotherapy. Similar patterns are observed for late-phase responses in the nose and airways [43].

Specific immunotherapy also induces allergen-specific IgG antibodies, particularly antibodies of the IgG4 subclass. At one time it was believed that these antibodies might intercept the allergenic particles at the mucosal surface and "block" the allergic response. While accepting that IgG antibodies are stimulated, their significance has been disputed, partly because the rise in IgG follows rather than precedes clinical benefit, and partly because many mast cells are on the mucosal surfaces, and therefore meet allergen before antibodies could interpose themselves. Moreover, there is a poor correlation between the amount of allergen-specific IgG and clinical protection. In most studies IgG correlates better with the dose of allergen that has been given, rather than with the degree of protection achieved. There has, however, been a recent resurgence of interest in a possible inhibitory role of specific IgG antibodies in grass pollen immunotherapy [44]. In particular, the time course of this effect suggests that specific IgG antibodies may interfere with IgE-dependent cytokine secretion from mast cells or prevent facilitated antigen presentation to T cells.

Allergen-specific T cell responses are also affected by specific immunotherapy. In nasal and skin allergen challenge models, successful specific immu-

NOTHERAPY is accompanied by a reduction in T cell and eosinophil recruitment in response to allergen. In parallel, there is a shift in the balance of Th1 and Th2 cytokine expression in the allergen-challenged site. Th2 cytokine expression is not affected but there is an increased proportion of T cells expressing the Th1 CYTOKINES IL-2, IFN-γ and IL-12 [45–47]. SPECIFIC IMMUNOTHERAPY with inhalant ALLERGENS induces regulatory T cells, which produce IL-10 and TGF-β [48, 49]. Among other actions, IL-10 stimulates production of the IgG4 subclass, which suggests that the IgG response after SPECIFIC IMMUNOTHERAPY may be a marker of the immune response, rather that the PRECISE mode of action conferring clinical benefit [50]. Taken together, these findings support the idea that SPECIFIC IMMUNOTHERAPY has a modulatory effect on allergen-specific T cells, and this may be why the clinical and late-phase responses to allergen challenge are attenuated without there being a substantial change in allergen-specific IgE ANTIBODY levels.

Specific immunotherapy for asthma

While the PRECISE mechanisms involved remain uncertain, there is a substantial body of clinical evidence and practice to support the use of SPECIFIC IMMUNOTHERAPY to treat allergic ASTHMA. However, the introduction of more effective inhaled therapies and the increased perception of side effects have led to a gradual decline in the number of patients receiving SPECIFIC IMMUNOTHERAPY for this indication. In some countries, most notably the UK, concern about adverse reactions has led to restrictions on the use of SPECIFIC IMMUNOTHERAPY for ASTHMA, although this remains a valid indication for SPECIFIC IMMUNOTHERAPY in North America and continental Europe [51, 52] (Tab. 3).

The EFFICACY of SPECIFIC IMMUNOTHERAPY in adult ASTHMA has been assessed in many trials over the last 65 years. The results of these studies have often been difficult to interpret, either because poor quality allergen extracts were used or because of poor study design. Many trials were not placebo-controlled; they were either open or single blind, and in most cases, only small numbers of patients were treated. A systematic review [52] identified 75 papers published between 1954 and 2001; 36 of these were for mite ALLERGY, 20 for pollen ALLERGY, 10 for animal dander ALLERGY, 2 for mould ALLERGY, 1 for latex ALLERGY and 6 for combinations of ALLERGENS. Concealment of allocation to placebo or active therapy was adequate in only 15 trials. A wide variety of different measurements were made, which makes it difficult to comment on the overall effectiveness of SPECIFIC IMMUNOTHERAPY. Symptom scores improved in the treated groups – it was necessary to treat four patients to prevent one from exacerbating, and to treat five patients to prevent one from needing an increase in medication. SPECIFIC IMMUNOTHERAPY reduced the airways response to inhalation of specific allergen and also improved non-specific bronchial reactivity.

Double-blind placebo-controlled studies have shown that SPECIFIC IMMUNOTHERAPY has a beneficial

TABLE 2. POSSIBLE MECHANISMS OF SUCCESSFUL SPECIFIC IMMUNOTHERAPY

Pharmacological desensitisation (short-term)
Abrogation of late-phase allergic responses
Induction of allergen-specific "blocking" antibodies
Induction of allergen-specific Th1 response
Induction of allergen-specific regulatory T cells
T cell anergy (cannot be overcome by exogenous IL-4)
"Mucosal tolerance"

TABLE 3. CURRENT INDICATIONS FOR SPECIFIC IMMUNOTHERAPY

Anaphylaxis to wasp/bee stings
Allergic rhinitis poorly controlled on standard drug therapy
Allergic asthma if mild and clearly allergic

effect in patients with grass pollen ASTHMA as assessed by a reduction in ASTHMA symptom and treatment scores. Active treatment led to a 60–75% reduction in symptom scores as compared to placebo-treated patients. An important study of SPECIFIC IMMUNOTHERAPY for ragweed ALLERGY found that patients who received active injections had an improvement in peak flow rates during the pollen season as well as reduced hay fever symptoms and reduced SENSITIVITY to laboratory challenge with ragweed pollen extracts [53]. In addition, the active group required much less anti-ASTHMA medication. However, the parallel economic analysis indicated that the cost-saving in ASTHMA drugs was less than the costs of SPECIFIC IMMUNOTHERAPY.

SPECIFIC IMMUNOTHERAPY also works in asthmatic patients sensitive to cats, where it reduces both the early ASTHMATIC RESPONSE to inhaled allergen and responses to simulated NATURAL exposure. However, SPECIFIC IMMUNOTHERAPY did not protect against allergen-induced increases in non-specific bronchial hyperresponsiveness. Other studies have reported reductions in both specific bronchial reactivity (to cat dander) and non-specific reactivity (to HISTAMINE) after SPECIFIC IMMUNOTHERAPY for cat ALLERGY [54].

The main drawback in using SPECIFIC IMMUNOTHERAPY to treat ASTHMA is the risk of serious adverse reactions. The vast majority of fatal reactions to SPECIFIC IMMUNOTHERAPY have occurred in patients with ASTHMA [55–57]. The incidence of systemic reactions in patients receiving SPECIFIC IMMUNOTHERAPY for ASTHMA varies between series and has been reported to range from 5% to 35%. So while ASTHMA is not an absolute contraindication, it is clear that patients with unstable ASTHMA should not be offered SPECIFIC IMMUNOTHERAPY, and caution should be exercised in anyone with an increased level of ASTHMA symptoms or transiently reduced peak flow rates.

Comparison of specific immunotherapy with other types of treatment for asthma

The majority of clinical trials of SPECIFIC IMMUNOTHERAPY for ASTHMA have compared SPECIFIC IMMUNOTHERAPY either with historical controls or with a matched placebo-treated group. To date, the effectiveness of SPECIFIC IMMUNOTHERAPY in ASTHMA has rarely been compared with conventional management (with avoidance measures and conventional inhaled or oral drugs). One study assessed SPECIFIC IMMUNOTHERAPY in asthmatic children receiving conventional drug therapy and found no additional benefit in patients who were already receiving optimal drug therapy [58]. There are some significant criticisms of this study and further work of this type is urgently needed. It is also important that trials include analysis of cost-benefit and cost-effectiveness since purchasers of health care are increasingly demanding this evidence before agreeing to fund therapies.

Effects on natural history of allergic disease

Children often start with a limited range of allergic sensitivities and progress over time to develop IgE against a wider range of inhaled ALLERGENS. Treatment with SPECIFIC IMMUNOTHERAPY may limit this tendency to acquire new sensitisations [59], although the clinical benefit of this preventive effect is not clear. It has also been suggested that SPECIFIC IMMUNOTHERAPY may modify the NATURAL history of ASTHMA in children who are known to be atopic but have not yet developed ASTHMA. In the key study, a group of 205 children aged 6–14 years, without previously diagnosed ASTHMA, were treated with SPECIFIC IMMUNOTHERAPY for birch or grass pollen ALLERGY in an open randomised design. Three years after completing treatment 45% of the untreated group had developed ASTHMA, while only 26% of the treated group had ASTHMA. These results have been sustained out to 7 years after completing therapy. Thus, four children had to be treated to prevent one case of ASTHMA, which makes this an extremely effective therapy [60].

SPECIFIC IMMUNOTHERAPY may also modify the progression of established ASTHMA. An early open study using uncharacterised mixed allergen extracts supported this view, with about 70% of treated chil-

dren losing their ASTHMA after 4 years of therapy, compared to about 19% of untreated controls, a result which was sustained up to the age of 16 years. The proportion of children whose ASTHMA was severe at age 16 was also much lower in the treated group [61]. By modern standards, this study was not well designed, and it needs repeating with modern SPECIFIC IMMUNOTHERAPY extracts in an up-to-date trial design.

In contrast, there is no current evidence that SPECIFIC IMMUNOTHERAPY influences the evolution of established ASTHMA in adults. Studies that have investigated withdrawal of therapy have found rapid recurrence of ASTHMA symptoms, although rhinitis symptoms seem to show much more sustained relief after SPECIFIC IMMUNOTHERAPY [62].

Thus, SPECIFIC IMMUNOTHERAPY is a valid but controversial treatment for ASTHMA. While it seems entirely logical to try to treat allergic disorders by specifically suppressing the immune response to the triggering agents, it remains unclear whether specific ALLERGY is important in all asthmatic patients who are sensitised, as judged by skin tests. Moreover, we remain unsure whether SPECIFIC IMMUNOTHERAPY in its present form is the best option for managing patients with allergic ASTHMA. We await proper comparative studies of best current SPECIFIC IMMUNOTHERAPY *versus* best current drug therapy, with robust endpoints including symptoms, objective measures of lung function, evaluation of cost-benefit ratios, safety, and quality of life. *In vitro* and *in vivo* measures such as skin test responses or allergen-specific IgG4 measurements are not sufficiently specific or sensitive to serve as surrogates for clinical EFFICACY. To date there have been relatively few well-controlled studies of SPECIFIC IMMUNOTHERAPY in ASTHMA but there is increasing evidence that SPECIFIC IMMUNOTHERAPY is beneficial in mite-induced and pollen-induced ASTHMA. The clinical EFFICACY of SPECIFIC IMMUNOTHERAPY in adult asthmatic patients sensitive to cats or moulds is less certain, and no comparative studies with conventional treatment have been performed. Further clinical trials are indicated, particularly in mild to moderate childhood ASTHMA and also in patients with atopic disease who have not yet developed ASTHMA but are at high risk of progression to ASTHMA.

Future directions

It must be acknowledged that there is scope to improve on current forms of SPECIFIC IMMUNOTHERAPY. It is hoped that developments in molecular biology will lead the way to improvements in both safety and EFFICACY. Possible avenues include the use of RECOMBINANT ALLERGENS, which would simplify the standardisation of allergen vaccines, and might allow fine tuning of vaccines for patients with unusual patterns of reactivity. Most allergic patients react to the same components of an allergen extract, the so-called major ALLERGENS, which are defined as those ALLERGENS recognised by over 50% of sera from a pool of patients with clinically significant ALLERGY to the material in question. However, not all patients recognise all major ALLERGENS and some patients only recognise ALLERGENS that are not recognised by the majority of allergic patient sera. This latter group may not respond to standard extracts, but might be better treated by a combination of ALLERGENS to which they are sensitive. Now that RECOMBINANT ALLERGENS for SPECIFIC IMMUNOTHERAPY are available, the range of sensitivities can be better characterised, and this may lead to patient-tailored vaccine products. Thus far, clinical trials have confirmed the EFFICACY of RECOMBINANT allergen cocktails but have not yet shown superiority to conventional vaccines [63]

RECOMBINANT molecular technology has also made it possible to develop novel forms of allergenic molecules. One group has developed a RECOMBINANT trimer consisting of three covalently linked copies of the major birch pollen allergen, Bet v 1. This trimer exhibited profoundly reduced allergenic activity even though it contained the same B cell and T cell EPITOPES as the native molecule, and was able to induce Th1 cytokine release. Interestingly, the rBet v 1 trimer induced IgG ANTIBODIES, analogous to the ANTIBODY response to standard SPECIFIC IMMUNOTHERAPY [64, 65]. Folding variants and other modifications of the physical structure of ALLERGENS may also improve the safety of SPECIFIC IMMUNOTHERAPY [66].

Since the B cell EPITOPES recognised by IgE molecules are usually three dimensional, whereas T cell EPITOPES are short linear peptide fragments of the antigen, it should be possible to use peptide

fragments of ALLERGENS to modulate T cells without risking ANAPHYLAXIS. Two distinct approaches have been tested. Either large doses of NATURAL sequence peptides are given, deceiving the T cell into high-dose TOLERANCE [67], or else an altered peptide LIGAND can be given. Both approaches require adjustment to the MHC type of the individual undergoing treatment. By sequential alteration of peptide sequences from the major ALLERGENS of house dust mite (*Dermatophagoides pteronyssinus*), it is possible to suppress proliferation of T cell CLONES recognising native house dust mite peptides, as well as suppressing their expression of CD40 LIGAND and their production of IL-4, IL-5 and IFN-γ. These anergic T cells do not provide help for B cells to switching class to IgE, and importantly this anergy cannot be reversed by providing exogenous IL-4 [68].

In an animal model, intranasal application of genetically produced hypoallergenic fragments of Bet v 1 produced mucosal TOLERANCE with significant reduction of IgE and IgG1 ANTIBODY responses, as well as reduced cytokine production *in vitro* (IL-5, IFN-γ, IL-10). These reduced immunological responses were accompanied by inhibition of the cutaneous and airway responses that were seen with the complete Bet v 1 allergen. The mechanisms of IMMUNOSUPPRESSION seemed to be different for the allergen fragments and the whole molecule, in that TOLERANCE induced with the whole Bet v1 molecule was transferable with spleen cells, whereas that induced by the fragments was not [69].

From epidemiological and experimental studies, we know that vaccination with mycobacteria has anti-allergic properties. In Japan, early vaccination with BCG was associated with a substantial reduction in the risk of developing ALLERGY [70], although similar associations were not evident in Sweden [71]. In animals, administration of BCG before or during sensitisation to ovalbumin reduces the degree of airway eosinophilia that follows subsequent challenge with ovalbumin. This effect is not mediated through any direct effect on IgE production, or blood eosinophil numbers, but is mediated through IFN-γ and can be reversed by exogenous IL-5 [72].

Two new approaches using DNA vaccines are also undergoing serious consideration. The first of these is a general approach, using CpG oligodeoxynucleotides (ODN). CpG ODN mimic bacterial DNA, and stimulate Th1-type cytokine responses. This approach exploits the ability of the innate response IMMUNE SYSTEM to recognise bacterial infection and respond vigorously [73]. In a mouse model of ASTHMA, pre-administration of CpG ODN prevented both airways eosinophilia and bronchial hyperresponsiveness [74]. Moreover, these effects were sustained for at least 6 weeks after CpG ODN administration [75]. However, clinical trials of non-specific CPG-based vaccines have not proved effective. An alternative approach is to couple CpG ODN to the allergenic protein, which enhances immunogenicity in terms of eliciting a Th1-type response to the allergen, but reduces its allergenicity [76]. *In vitro*, this coupled vaccine stimulates Th1 cytokine expression in cultured human peripheral blood mononuclear cells [77]. Initial clinical trials confirmed that the hybrid vaccine elicits a Th1-pattern response [78], but subsequent trials were inconclusive. Anti-sense DNA vaccines could also be used to down-regulate airway adenosine RECEPTORS. In animal models of ASTHMA this leads to a reduction in allergen-induced airway responses [79]. A contrasting approach is to use allergen-specific naked DNA sequences as vaccines. This technology is still in its infancy, but preliminary data suggest that giving naked DNA leads to production of ALLERGENS from within the airways epithelial cells [80, 81]. Due to the different handling pathways for endogenous and exogenous ALLERGENS, it seems that the endogenously produced allergen elicits a Th1-type response and if this can be reproduced in allergic humans, it is hoped that this may overcome the existing Th2-pattern response and eliminate the ALLERGY. Initial animal studies have confirmed the concept and show that the DNA vaccine prevents sensitisation upon subsequent immunisation with the allergen [82]. However, the potential for generating a powerful Th1-type response to ubiquitous agents means that this approach will need further, careful evaluation in animal models before it can be pursued in man.

Conclusions

SPECIFIC IMMUNOTHERAPY has been in use for over a century, and is clinically effective in patients with

rhinitis or ASTHMA whose symptoms are driven by allergic triggers. It is perhaps surprising that we still do not know exactly how SPECIFIC IMMUNOTHERAPY works, but the general view is that the vaccination protocol induces regulatory T cells that dampen the response to allergen exposure in sensitised subjects.

Over time this response alters, suggesting that a more permanent state of TOLERANCE may be achieved by the long course of desensitisation [83].

When used in appropriately selected patients, SPECIFIC IMMUNOTHERAPY is effective and safe, but care is needed to recognise and treat adverse reactions. As well as careful patient selection, care is needed to minimise the risks of side effects. Appropriate training of allergists and clinic support staff is an essential element of ensuring safety. Future directions in SPECIFIC IMMUNOTHERAPY will include the development of better standardised vaccines, and the use of RECOMBINANT ALLERGENS, both of which should improve the safety profile of SPECIFIC IMMUNOTHERAPY. In parallel, the development of allergen-independent immunomodulatory therapies may allow more general approaches to be developed, which would be particularly advantageous for those patients who are sensitised to multiple ALLERGENS.

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Immunostimulants in cancer therapy

James E. Talmadge

Introduction

Immunoaugmenting agents have been used to treat disease since William B. Coley treated cancer patients with mixed bacterial toxins early in the 20th century [1]. These early studies spawned the clinical use of such microbially derived substances as Bacille Calmette-Guerin (BCG) (bladder cancer, USA), krestin, picibanil and LENTINAN (gastric and other cancers, Japan), and Biostim and Broncho-Vaxom (recurrent infections, Europe). While these "crude" drugs induce numerous immunopharmacological activities, they pose considerable regulatory obstacles due to impurity, lot-to-lot variability, unreliability and adverse side effects. Similarly, traditional herbal medicines (Asia) also provide a source of active substances for IMMUNOTHERAPY. The purification, characterization and SYNTHETIC production of the active moieties from NATURAL products (Bestatin, Taxol) and culture supernatants (FK-506, rapamycin, deoxyspergualin and CICLOSPORIN) have also provided valuable drugs. The current focus within IMMUNOTHERAPY is on the use of RECOMBINANT proteins (CYTOKINES), although the utility of these drugs can be limited due to BIOACTIVITY and pharmacological deficiencies. Thus, there remains a potential role for classical BIOLOGICAL RESPONSE MODIFIERS (BRMs) due to their oral BIOAVAILABILITY and ability to induce multiple CYTOKINES for immune augmentation and hematopoietic restoration.

In 2002, the 20th anniversary of the first approved biopharmaceutical, RECOMBINANT insulin (Humulin: Genentech, South San Francisco, CA, USA), was observed. Today, biotechnological drugs incorporate not only immunoregulatory proteins, enzymes and BIOLOGICALS derived from NATURAL sources, but also engineered (manipulated) MONOCLONAL ANTIBODIES (mAbs) and CYTOKINES, in addition to GENE THERAPY and TISSUE ENGINEERING strategies [2]. In 2008, there were 250 BIOPHARMACEUTICALS approved in the United States and or European Union (EU) for use in humans with 174 approved between January 1995 and June 2007 [2]. In addition, some 400 BIOLOGICALS are currently undergoing clinical evaluation [3]. Perhaps the most telling statement regarding the maturation of the field is that the European Union (EU), as of March 2005, had approved six biosimilars. These are BIOLOGICALS that are comparable in quality, safety, and EFFICACY to a reference product [4, 5].

The overall approach in this chapter is to limit the discussion of BIOTHERAPEUTICS to the RECOMBINANT, NATURAL and SYNTHETIC drugs that are currently approved for clinical use against cancer (Box 1). We have focused on RECOMBINANT proteins (except antithrombotics, VACCINES and mAbs) and do not discuss nucleic acid-based or tissue engineered gene therapeutic products. A brief discussion follows regarding COMBINATION THERAPY and CELLULAR THERAPY as future prospects.

Recombinant proteins

RECOMBINANT proteins have emerged as an important class of drugs for the treatment of cancer, IMMUNOSUPPRESSION, myeloid dysplasia and infectious diseases. However, our limited understanding of their pharmacology and mechanism of action (MOA) has hindered their development (Tab. 1). To facilitate advancement, information is needed on their pharmacology, MOA and toxicology [6, 7]. One approach to the development of BIOTHERAPEUTICS is to identify a CLINICAL HYPOTHESIS based upon therapeutic SURROGATE(s) identified during preclinical pharmacological studies [8, 9]. A

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Box 1. Types of cancer amenable to immunotherapy

- Melanoma and renal carcinoma. These tumors are highly antigenic and frequently have a significant histocytic and lymphocytic cellular infiltration. As such, they have demonstrated significant responsiveness to intervention with biotherapeutics.
- Bladder cell carcinoma. Superficial transitional cell carcinoma has proven highly responsive to therapy with BCG as well as cytokines such as interferon-α (IFN-α).
- Head and neck cancer. Head and neck cancer is responsive to cytokines therapy, due in part to its availability for therapeutic intervention and has been shown to be highly responsive to low dose paralymphatic administration of interleukin-2 (IL-2).
- Solid tumors. A number of solid tumors have shown a response to immune augmenting agents, but in general, phase III trials have not been undertaken to demonstrate responsiveness.
- Leukemias and lymphomas. In general, these 'liquid' tumors have shown responsiveness to several immune augmenting agents, including intervention with IFNs.

surrogate for clinical efficacy may be a phenotypical, biochemical, enzymatic, functional (immunological, molecular or hematological) or quality-of-life measurement that is believed to be associated with therapeutic activity. Phase I clinical trials can then be designed to identify the optimal immunomodulatory dose (OID) and treatment schedule that maximizes the augmentation of surrogate end point(s). Subsequent phase II/III trials can be established to determine if the changes in the surrogate levels correlate with therapeutic activity.

Table 1 lists the immunologically and hematologically active cytokines that are approved for use in the United States. This information is expanded in Appendix 1.

In contrast to strategies based on the identification of surrogates for therapeutic efficacy, protocols for recombinant proteins are often identified based on practices developed for conventional drugs. However, these may not be advantageous for the identification of therapeutic efficiency in response to cytokines (Box 2).

Box 2. Pharmacological and dose-relationship considerations with cytokines

Cytokines have in several instances shown increased bioactivity following delivery by slow release [243–245]. The covalent attachment of polyethylene glycol (PEG) to cytokines (pegylation) (Tab. 2), including IFN-α and granulocyte colony-stimulating factor (G-CSF), has significant enhanced biological activity due, in part, to their improved pharmacokinetic profile [244, 245]. Thus, strategies to limit pharmacological deficiencies are critical to the development of recombinant biotherapeutics. The pharmacological attributes of recombinant biotherapeutics are improved with targeted delivery, which prolongs their short half-life [246]. In addition, there can be unexpected relationships between the dose administered and the biological effect of recombinant biotherapeutics, including a non-linear-dose relationship, described as "bell-shaped" [38, 247]. This lack of a linear, dose-response relationship may be due to non-linear dispersal throughout the body, poor ability to enter into a saturable receptor-mediated transport process, chemical instability, sequence of administration with other agents, an incorrect time of administration, inappropriate location, and/or response of the target cells. A "bell-shaped" dose-response curve may be associated with receptor tachyphylaxis expression or a signal transduction mechanism whereby the cells become refractory to subsequent receptor-mediated augmentation.

Table 1. Drugs with multiple targets

Drug	Indications	Approval date
Aranesp	Anemia caused by chemotherapy and anemia associated with chronic renal disease	June 01
Bone morphology protein-2	Treatment of spinal degenerative disc disease	July 02
Denosumab	Osteoporosis, mAb to RANK ligand	February 09
IFN-βIb	Treatment of relapsing multiple sclerosis	August 09
IL-2	CTCL, metastatic melanoma, renal cell carcinoma	May 92
IFN-α	Treatment of relapsing multiple sclerosis, chronic hepatitis C viral infection, AIDS-related Kaposi's sarcoma, follicular lymphoma, genital warts, hairy cell leukemia, hepatitis B and C, malignant melanoma.	May 96
IFN-γ	Management of chronic granulomatous disease and osteopetrosis.	December 90
IFN	Treatment of hepatitis C	March 99
LFA-1/IgG1	Moderate to severe chronic plaque psoriasis	January 03
rEPO	Anemia caused by chemotherapy, anemia, chronic renal failure, anemia in Retrovir®-treated HIV-infected, chronic renal failure, dialysis. Surgical blood loss.	June 89
rG-CSF	Acute myeloid leukemia, autologous or allogeneic bone marrow transplantation, chemotherapy-induced neutropenia, chronic severe neutropenia, peripheral blood progenitor cell transplantation.	February 91
rHPDGF-BB	Diabetic neuropathy, foot ulcers	1997
rHu IL-11	Chemotherapy-induced thrombocytopenia	November 97
rHuGM-CSF	Allogeneic and autologous bone marrow transplantation, neutropenia resulting from chemotherapy, peripheral blood progenitor cell mobilization.	March 91
Stem Cell Factor	Mobilization of hematopoietic stem cells	1997
TNFR:Fc	Moderate to severe active rheumatoid arthritis and juvenile rheumatoid arthritis, active ankylosing spondylitis.	November 98

AIDS, acquired immune deficiency syndrome; CTCL, cutaneous T cell lymphoma; RANK, receptor activator of nuclear factor kappa-beta; RCC, renal cell carcinoma

Interferon-α

Clinical activity against cancer

The initial, non-randomized, clinical studies with interferon (IFN)-α suggested that it had therapeutic activity for malignant melanoma, osteosarcoma and various lymphomas [10]. Subsequent randomized trials, however, demonstrated significant therapeutic activity against only less common tumor histiotypes, including hairy cell and chronic myelogenous leukemia (CML) [10–12], and a few types of lymphoma [12], including low-grade non-Hodgkin's lymphoma [13] and cutaneous T cell lymphoma [14]. Currently, the list of responding indications has expanded to include malignant melanoma [15], acquired immune deficiency syndrome (AIDS) and Kaposi's sarcoma [16], genital warts, and hepatitis B and C.

Pharmacological actions – Dose response

It has taken almost three decades to translate the concept of IFN-α as an anti-viral to its routine utility in clinical oncology and infectious diseases. Despite extensive study, the development of IFN-α is still in its early stages, and basic parameters, such as optimal dose and therapeutic schedule, remain to be determined [11, 12, 17]. The MOA is also controversial since IFN-α has been shown to have dose-dependent antitumor activities *in vitro*, yet be active at low doses for hairy cell leukemia [11, 12]. IMMUNOMODULATION as the mechanism of therapeutic activity of IFN-α is perhaps best supported by its action against hairy cell leukemia. Treatment with IFN-α is associated with a 90–95% response rate. However, this is not fully achieved until the patients have been on the protocol for a year, and it appears that low doses of IFN-α are as active as higher doses [18]. It should be noted that the clinical use of IFN-α has been supplanted by other even more effective drugs, but its approval for hairy cell leukemia precipitated expanded studies of IFN-α for other diseases.

The initial dose-finding studies determined that a dose of IFN-α of 12×10^6 U/m^2 was not tolerable in patients with hairy cell leukemia [12]. Subsequently, it was demonstrated that highly purified NATURAL IFN-α (2×10^6 U/m^2) was both well tolerated and effective when administered three times per week for 28 days [19]. However, it retained some toxicity, including myelosuppression as well as neurotoxicity and cardiotoxicity. In these studies, a lower dose (2×10^5 U/m^2) was also administered for 28 days and found to be better tolerated than the standard dose, while inducing equivalent improvements in neutrophil and platelet counts. Further, substantial clinical improvement (increased platelet and neutrophil counts) was observed within the first 4–8 weeks of treatment as well as an improved quality of life (decreased cardiac and neurologic toxicity, myelosuppression, flu-like syndrome, platelet transfusions, and bacterial infection incidences). However, IFN-α also generates a therapeutic dose-response effect, whereby higher doses of IFN-α will induce a quantitatively greater anti-leukemic response than that observed with low doses of IFN-α. Thus, therapy with IFN-α is initially at 2×10^5 U/m^2, allowing patients to become tolerant to the acute toxicity of IFN-α, with a subsequent dose increase to 2×10^6 U/m^2 to obtain a greater anti-leukemic effect. In CML, sustained therapeutic responses are found in more than 75% of patients [20, 21]. In addition to reducing leukemic cell mass, there is also a gradual reduction in the frequency of cells bearing a 9-22 chromosomal translocation [22] (Box 3).

The unique cellular and molecular activities of IFN-α can potentially complement the MOA of other therapies [23]. At present, therapeutic applications for IFN-α are focused on synergistic or additive effects with IFN-γ, granulocyte-monocyte colony-stimulating factor (GM-CSF) and INTERLEUKIN (IL)-2. The MOA of IFN-α, as well as the optimal dose, remains controversial. A recent meta-analysis of 12 clinical studies for high-risk melanoma showed a significant recurrence-free survival (RFS) follow-

BOX 3. GLEEVEC

Until the introduction of Imatinib mesylate, IFN-α was the standard treatment for patients in chronic phase CML. Imatinib mesylate (STI571, Gleevec), a signal transduction inhibitor, has demonstrated significant activity, including molecular responses as a single agent for the treatment of CML. Patients who failed IFN-α therapy are responsive to Imatinib. Therefore, at present, it is used either as first line monotherapy or to rescue patients who have failed IFN-α therapy [197]. In addition to its activity in chronic phase CML, Gleevec also has therapeutic activity for patients with accelerated and blast phase CML [248]. Long-term survival and apparent cure are also achieved with allogeneic stem cell transplantation (SCT) for CML patients who have a donor and who can tolerate the transplant [249, 250]. Phase III studies using Gleevec have shown that it can induce major molecular responses, complete cytogenetic responses with a significantly more rapid induction of molecular responses in patients receiving higher doses as compared to lower doses [251].

ing treatment with IFN-α. However, the benefit of IFN-α therapy on overall survival (OS) is less clear [24]. There is a significant trend towards a correlation between increasing dose and RFS, but not OS. Similarly, studies on immune augmentation in melanoma patients receiving high- or low-dose IFN-α revealed no association between immune response and baseline phenotypical and functional immunity. However, numerous immune surrogates are augmented by IFN-α treatment and are associated with dosage. Administration of IFN-α has been shown to significantly up-regulate MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) class II and intercellular ADHESION MOLECULE (ICAM)-1 expression on tumor cells in a dose-dependent manner. In addition, NATURAL killer (NK) cell and T cell functions are augmented in a dose-dependent manner by IFN-α, as are changes in T cell phenotypes. Further, high-dose IFN-α regulates immune parameters more rapidly than low-dose IFN-α. However, in this study of 51 high-dose, 54 low-dose and 43 no-IFN-α patients, there was no relationship between immune augmentation and RFS [25].

Interferon-γ

Effect in animal models and MOA

IFN-γ has multiple, potential mechanisms that may be involved in tumor protection and therapy. These include, but are not limited to: (1) anti-proliferative activity for tumor growth/survival; (2) induction of ANGIOSTASIS; and (3) augmentation of both innate and ADOPTIVE IMMUNITY (Fig. 1). However, it is unclear

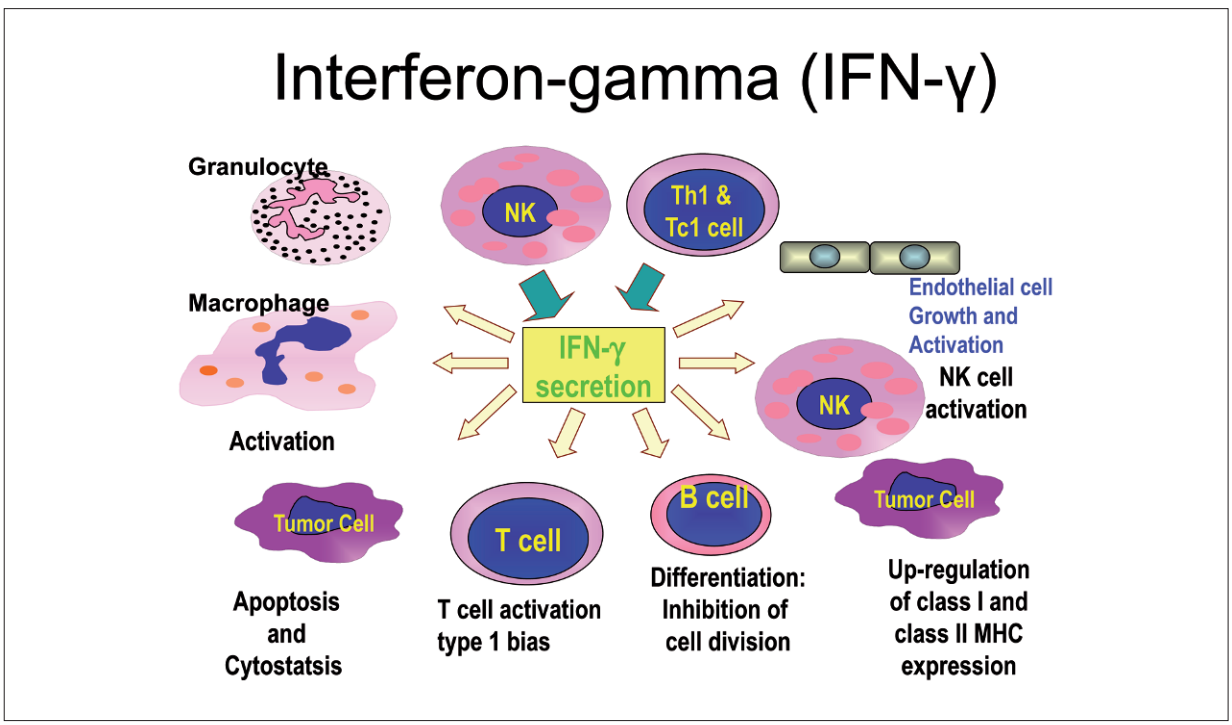

FIGURE 1. REGULATION OF IMMUNE RESPONSES BY INTERFERON-γ (IFN-γ)
IFN-γ is produced predominately by T helper cell type 1 (Th1), T cytotoxic cell type 1 (Tc1) and natural killer (NK) cells, resulting in the activation of T cells, NK cells, macrophages, and granulocytes. In addition, it up-regulates major histocompatibility complex (MHC) class I and class II expression on tumor cells as well as the expression of a wide variety of receptors on both tumor cells and epithelial cells.

which, if any, of these potential mechanisms are critical for the activity of this pleiotropic cytokine.

Anti-proliferative effects on tumor growth/survival

IFN-γ has direct anti-proliferative and anti-metabolic effects on tumor cells. It can also induce the APOPTOSIS of tumor cells *via* conventional signaling mechanisms, resulting in the induction of genes that promote cellular APOPTOSIS, including caspase-1 (IL-1α converting enzyme or ICE) [26] and Fas LIGAND (FasL) [27].

Induction of angiostasis

The anti-tumor activity of IFN-γ can also be mediated by an inhibition of NEO-ANGIOGENESIS. The growth of solid tumors requires new blood vessel formation, which is a result of tumor-induced angiogenesis [28]. Pro-angiogenic molecules are secreted by tumors, including vascular endothelial cell growth factor (VEGF) and basic fibroblast growth factor (bFGF). However, IFN-γ induces CHEMOKINES with potent angiostatic actions, including inducible protein-10 (IP-10) [29]. In addition, IP-10 belongs to a family of CXC non-ELR (enzyme-linked receptor) CHEMOKINES that all have angiostatic activity and whose expression is regulated by IFN-γ.

Augmentation of both innate and adoptive immune responses

IFN-γ is a potent macrophage-activating cytokine capable of augmenting macrophage-mediated tumoricidal activity *in vitro* and *in vivo* [30,31]. IFN-γ-activated MACROPHAGES express multiple tumoricidal mechanisms, including the production of reactive oxygen and/or nitrogen intermediates and up-regulated expression of CYTOTOXIC ligands including TUMOR NECROSIS FACTOR (TNF)-α, FasL [32], and TNF-related APOPTOSIS-inducing LIGAND (TRAIL) [33]. In addition, IFN-γ significantly enhances IL-12 secretion by MACROPHAGES and DENDRITIC CELLS (DCs) [34]. NK cells also have a potential role in promoting anti-tumor responses *via* at least two mechanisms. NK cells are important sources of IFN-γ and also exert direct cytocidal activity against tumors through mechanisms involving perforin [35] and TRAIL [36]. In addition, IFN-γ can markedly enhance adaptive T cell responses to tumors. IFN-γ also has an important role in regulating the Th1/Th2 balance during the host response to a tumor [36].

Dose-response relationship

Preclinical studies have suggested that IFN-γ has significant therapeutic activity with a "BELL-SHAPED" dose-response curve [37]. Studies of immune response in normal animals have revealed the same "BELL-SHAPED" dose-response curve for the augmentation of macrophage tumoricidal activity [19, 37]. Thus, optimal therapeutic activity is observed with the same dose and protocol of IFN-γ but with significantly less therapeutic activity at lower and higher doses. A significant correlation between macrophage augmentation and therapeutic EFFICACY has been reported [19], suggesting that immunological augmentation provides one mechanism for the therapeutic activity of IFN-γ, and supports the hypothesis that treatment with the maximum tolerated dose (MTD) of IFN-γ may not be optimal in an adjuvant setting. The preclinical hypothesis of a "BELL-SHAPED" dose-response curve for IFN-γ has been confirmed in clinical studies on the immunoregulatory effects of IFN-γ, which defined an OID [38–40]. In general, the OID for IFN-γ has been found to be between 0.1 and 0.3 mg/m^2 [39]. In contrast, the MTD for IFN-γ may range from 3 to 10 mg/m^2, depending upon the source of IFN-γ and/or the clinical center. The identification of an OID for IFN-γ in patients with minimal tumor burden has resulted in the development of clinical trials to test the hypothesis that the immunological enhancement induced by IFN-γ will result in prolongation of the disease-free period and OS of patients in an adjuvant setting [40].

Clinical therapeutic activity – MOA

IFN-γ was found on an empirical basis to have therapeutic activity in chronic granulomatous disease (CGD) [41] and it was for this indication that the US Food and Drug Administration (FDA) first approved IFN-γ (Box 4). In addition to its approval for CGD, IFN-γ has also been approved for the treatment of RHEUMATOID ARTHRITIS (RA) in Germany and, very recently, severe malignant osteopetrosis in the

Box 4. IFN-γ in CGD

Studies in patients with CGD suggest that the mechanism of therapeutic activity by IFN-γ is associated with enhanced phagocytic oxidase and superoxide activity in polymorphonuclear cells (PMNs). However, more recent data suggests that the majority of CGD patients obtain clinical benefit by prolonging IFN-γ therapy and that the MOA may not be due to enhanced PMN oxidase activity, but rather the correction of a respiratory-burst deficiency in monocytes [252]. Further, IFN-γ administration could induce nitric oxide (NO) synthetase activity by PMNs in patients with CGD [256]. Following 2 days of IFN-γ administration, a significant increase in PMN-produced NO is observed in association with an increase in the bactericidal capacity of PMNs [253]. As PMNs in patients with CGD lack the capacity to produce superoxide anions, it is possible that the increased NO release and *in vitro* bactericidal activity are instrumental in augmenting host defenses and reducing the morbidity of CGD [253]. Similarly, IFN-γ increases PMN expression of Fc gamma receptor I (FcγRI) and improves FcγR-mediated phagocytosis, at least in normal subjects [254]. Therefore, the MOA of IFN-γ that is critical in reducing the frequency of infections in patients with CGD may not be increased oxidase and superoxide production, but rather effects on other mechanisms of granulocyte activity, including NO production.

United States. In a randomized phase III trial, IFN-γ was also reported to have activity in women receiving first-line platinum-based chemotherapy against ovarian cancer [42]. In this study, there was a significantly higher response rate and longer progression-free survival (PFS) in women receiving IFN-γ plus chemotherapy when compared to chemotherapy alone. However, there was no statistically significant improvement in OS. The IFN-γ MOA is unknown for this study, although the authors speculated that it may be associated with the inhibition of HER-2/neu expression. Currently, IFN-γ is being studied for the treatment of idiopathic pulmonary fibrosis [43].

Interleukin-2

Pharmacological actions relevant to cancer

IL-2, a T cell growth factor, has a significant role in regulating immunity to infectious and neoplastic diseases (Fig. 2). IL-2 is produced primarily by activated DCs, NK and T cells and induces pleiotropic biological responses after binding to one of three RECEPTORS (see also chapter A5). IL-2 stimulates the growth of naïve T cells following antigen (Ag) activation, and later induces activation-induced cell death (AICD) [44,45]. IL-2 also has effects on several other immune cells, including NK cells [46], B cells [47], monocyte/MACROPHAGES [48], and PMNs [49]. The ability of IL-2 to stimulate NK and T cell lysis of tumor cells stimulated clinical interest in IL-2 [50]. NK cells are part of the INNATE IMMUNE SYSTEM and comprise 10–15% of peripheral blood LYMPHOCYTES (PBL). Functionally, NK cells are characterized by NK and lymphokine-activated killing (LAK), ANTIBODY-dependent cellular CYTOTOXICITY (ADCC), and immunoregulatory cytokine production (see chapter A6). NK cells also express NK RECEPTORS (NKRs) that recognize MHC class I ligands and regulate, inhibit, and activate a response to TARGET cells [51].

One important *in vivo* role of IL-2 for T cell responses is the promotion of thymic development and peripheral expansion of $CD4^+CD25^+$ T cells known as T regulatory (Treg) cells. Loss of Treg activity in IL-2- or IL-2R-deficient mice results in lymphadenopathy and AUTOIMMUNE DISEASE. It appears that IL-2-dependent Treg cells regulate homeostatic and Ag-induced T cell proliferative responses, resulting in pathological autoreactivity [52]. IL-2 and its high-AFFINITY RECEPTOR are rapidly up-regulated following binding with a cognate Ag, and the absence of either IL-2 or IL-2R can result in a loss of immune reactivity [53]. Further, the induction of $CD8^+$ T cell responses is dependent upon the presence of $CD4^+$ T cell help and their secretion of IL-2 [54]. Recently, IL-2 production by DCs has also been shown to be essential for the initiation of both $CD4^+$ and $CD8^+$ T cell responses [55].

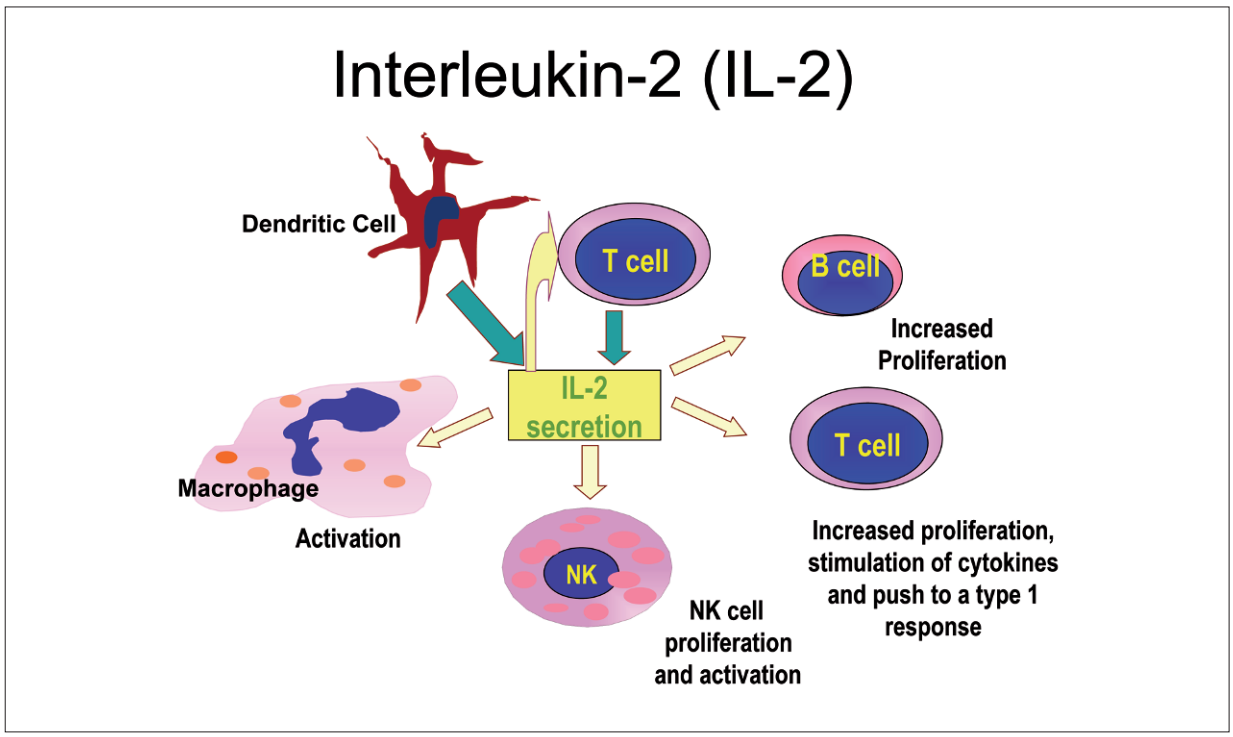

Figure 2. Regulation of immune responses by interleukin-2 (IL-2)
IL-2 production by T cells and dendritic cells (DCs) supports the proliferation of T cells, B cells, and NK cells, in addition to establishing a bias towards a Th1 cell response. IL-2 can also activate monocytes and NK cells, resulting in increased cytotoxicity.

IL-15 was identified based upon its ability to stimulate proliferation of IL-2-dependent T cells in the presence of neutralizing IL-2 Abs [56]. IL-15 shares two of the three IL-2Rs including IL-2/15Rβ and the γc receptor subunits [56]. Similar to IL-2, resting T cells do not respond to IL-15, and T cell Ag receptor (TCR) ligation induces IL-15Rα expression, allowing a response to IL-15. Both IL-2 and IL-15 can induce the proliferation of activated T cells and the differentiation of cytotoxic T lymphocytes (CTL) and LAK cells expressing IL-2R or IL-15R *in vitro*. However, despite their shared receptor usage, distinct roles for IL-2 and IL-15 are observed for the proliferation and survival of $CD4^+$ and $CD8^+$ T cells [45]. Both IL-2 and IL-15 promote the proliferation of $CD4^+$ T cells, but continued stimulation with IL-2 promotes AICD. IL-15 has the opposite effect and can even inhibit IL-2-induced AICD of $CD4^+$ T cells [57]. IL-15 can also selectively stimulate the proliferation of memory $CD8^+$ T cells, in contrast to IL-2, which inhibits $CD8^+$ memory T cell proliferation [58]. Thus, IL-15 and IL-2 have similar biological activity *in vitro*, while their critical and non-redundant functions *in vivo* are distinct [59, 60]. Further, IL-15 is better than IL-2 in mediating NK cell differentiation and promoting memory $CD8^+$ T cell survival. Given the role of IL-15 in the maintenance and proliferation of T cells, including memory $CD8^+$ T cells, a clinical investigation is likely [61].

Clinical effects against cancer

IL-2 has been approved for use as a single agent in the treatment of RCC, metastatic melanoma and

hepatitis C. It is also administered in conjunction with LAK or T cell infiltrating LYMPHOCYTES (TILs) in adoptive CELLULAR THERAPY protocols. TILs are T cells obtained from a tumor and when expanded *in vitro* with low levels of IL-2 and in the presence of tumor Ag result in a population of tumor-specific CYTOTOXIC T cells. However, it has been questioned whether the adoptive transfer of LAK or TIL cells is necessary or adds to the clinical EFFICACY of IL-2. Indeed, there has been little indication of an improved therapeutic effect of IL-2 plus LAK cells *versus* IL-2 alone [62, 63]. When the clinical trials with IL-2 are rigorously examined, neither strategy has impressive (as opposed to significant) therapeutic activity [63, 64]. The overall response rate with IL-2 is 7–14% and is associated with considerable toxicity [65]. However, it should be remarked that most of these responses are durable. In one of the first clinical studies [66], partial responses were observed in 4 out of 31 patients. Interestingly, these partial responders did not correspond to patients with increased LAK or NK cell activity. The antitumor effect of both TIL and LAK cells could be due to either a direct effect or secondary to the induction of other cytokine mediators. This is suggested by the observation that IL-2-stimulated LYMPHOCYTES produce IFN-γ and TNF as well as other CYTOKINES, and that the therapeutic activity of IL-2 may be synergistic with these CYTOKINES [66]. Recently, IL-2 has also been examined as an adjuvant to augment the tumor host response to HUMAN IMMUNODEFICIENCY VIRUS (HIV) [67] and anticancer VACCINES [68].

Many of the IL-2 clinical trials in metastatic RCC, with or without LAK cells, have used an MTD of IL-2. A study by Fefer et al. [69] compared maintenance IL-2 therapy at the MTD or a dose 60% lower. They found that it was possible to maintain patients on therapy for a median of 4 days at the IL-2 MTD, but with the appearance of severe hypertension and capillary-leak syndrome. In the lower dose protocol, none of the patients experienced severe hypertension or capillary-leak syndrome, and the median duration of maintenance IL-2 therapy was 9 days. Further, there was a total response rate of 41% in the lower dose protocol compared to a 22% response rate for the higher dose protocol and a shorter duration of administration. These investigators suggest that there may be an improved therapeutic activity associated with a longer IL-2 maintenance protocol that can be achieved at lower doses.

Dose response, toxicity and pharmacological studies

Several IL-2 dose-response studies have examined the affect of IL-2 administration on cytokine MESSENGER RIBONUCLEIC ACID (mRNA) levels in the PBL of cancer patients. The results from one IL-2 dose-response study suggested that: (1) doses of IL-2 as low as 3×10^4 U/day administration by continuous infusion could augment T cell function, and (2) doses of IL-2 $\geq 1\times10^5$ U/day increases not only T cell, but also macrophage function [70]. The latter was measured as an up-regulation of TNF mRNA, which was observed at the higher dose of IL-2. The increased TNF mRNA levels combined with the increased levels of IFN-α observed at the lower dose of IL-2 may be responsible for IL-2 toxicity [71]. The effect of low-dose, subcutaneous (s.c.) IL-2 administration was studied in healthy males at 1000 or 10 000 international units (IU)/kg [72]. No consistent changes were observed with 1000 IU/kg; however, phenotypic and immunoregulatory changes were observed following administration of 10 000 IU/kg IL-2. This dose significantly depressed the number of circulating LYMPHOCYTES, including $CD4^+$, $CD8^+$, and activated T, B, and NK cells. In contrast, the number of NEUTROPHILS and MONOCYTES were increased. There was also a significant increase in IL-4 serum levels, while the levels of IFN-γ and IL-2 RECEPTOR were significantly depressed. Kinetic studies revealed that these effects varied with time, but occurred at IL-2 serum levels sufficient to saturate the high-AFFINITY RECEPTOR and, by 3 hours following injection of 10 000 IU/kg IL-2, remained elevated for approximately 12 hours. Together, these studies suggest that IL-2-mediated immune augmentation can occur with low doses of IL-2 administered s.c. or by continuous infusion.

The potential for IL-2 to be active at low doses was also shown in patients with squamous cell carcinoma of the oral cavity and oropharynx [73]. In this study, 202 patients were randomly assigned to receive either surgery and radiotherapy or surgery, radiotherapy, and daily IL-2. The IL-2 was injected perilymphatically at 5000 U IL-2, daily for 10 days

prior to and following surgery. IL-2 was also given post surgery for 12-monthly cycles, each consisting of five daily injections. This study revealed a significant increase in disease-free survival (DFS) and OS in the IL-2-treated patients.

In a study of RCC patients, cohorts were randomized to receive either a high-dose intravenous (i.v.) IL-2 regimen or one using one-tenth the dose (72 000 IU/kg/8 h) administered by the same schedule [74]. A third arm using the low dose of IL-2, given daily by s.c. administration, was added later. In the most recent interim report [64], 156 patients were assigned to the high-dose arm, 150 patients to the low-dose i.v. arm and 94 patients to the low-dose s.c. arm. Toxicities were less frequent when IL-2 was given at the low doses, especially hypotension, but there were no IL-2-associated deaths in any arm. A higher response rate was observed in the patients given the high dose of IL-2 (21%) *versus* low dose i.v. IL-2 (13%), but no OS differences were observed. The response rate of s.c. IL-2 (10%) was similar to the low-dose i.v. IL-2, but was significantly different from high-dose IL-2 therapy ($p=0.033$). The response duration and survival in the complete responders was significantly better in patients receiving high-dose IL-2, as compared to low-dose IL-2 i.v. therapy ($p=0.04$). Thus, tumor regressions, as well as complete responses, were seen with all IL-2 regimes. Further, it was suggested that IL-2 is more clinically active at the higher dose given i.v., although this did not provide an OS benefit and only a small percentage of patients achieved durable clinical response.

Granulocyte-monocyte colony-stimulating factor

Pharmacological activity related to cancer therapy

GM-CSF was initially defined by its ability to support the growth of both granulocyte and macrophage colonies from hematopoietic precursor cells [75]. However, GM-CSF-deficient mice have no obvious deficiency in myeloid cells [76], suggesting the presence of redundant growth factor(s). GM-CSF can also potentiate the functions of mature GRANULOCYTES and MACROPHAGES [77], in addition to its role as a hematopoietic regulator (Fig. 3) [77].

Similar to other proinflammatory CYTOKINES, the production and activity of GM-CSF occur at the site of INFLAMMATION and increased levels of GM-CSF mRNA are observed in skin biopsies from allergic patients with cutaneous reactions [78] and in arthritic joints [79]. At present, GM-CSF is considered an important regulator (proliferation, maturation and activation) of granulocyte and macrophage lineage populations at all stages of maturation (see chapter A5).

GM-CSF in combination with TNF-α or IL-4 can differentiate MONOCYTES into DCs *in vitro* [80], which are then used as VACCINES. Treatment of mice with PEGYLATED GM-CSF has also been shown to expand DCs in the spleen [81], and clinically, the number of circulating DCs is expanded in the peripheral blood (PB) by GM-CSF and IL-4 administration [82].

Clinical use in cancer patients

GM-CSF was approved in 1991 by the FDA to support transplant associated NEUTROPENIA and to mobilize STEM CELLS. In Europe, it is also approved for prophylactic treatment following dose-intensive chemotherapy. However, the rate of absolute neutrophil count (ANC) recovery in patients receiving myelosuppressive chemotherapy or in the mobilization of stem cells into the PB in response to treatment with GM-CSF is 1 day slower than that observed with G-CSF. It also has a toxicity profile, including low grade fever, myalgias and bone pain that may be slightly greater than that observed with G-CSF. Because of the real and perceived problems, GM-CSF has been used to a lesser extent than G-CSF. However, patients receiving chronic GM-CSF therapy after marrow graft failure have been shown to have significantly improved survival in comparison to historically matched controls [83]. Similarly, significantly reduced hospital and antibiotic therapy duration have been reported for several phase II and III clinical trials [84]. Thus, patients undergoing autologous BONE MARROW transplantation (BMT) or STEM CELL TRANSPLANTATION (SCT) and receiving prophylactic GM-CSF administration have demonstrated significant improvements in ANC recovery, fewer cases of infection and fewer days spent in the hospital [85].

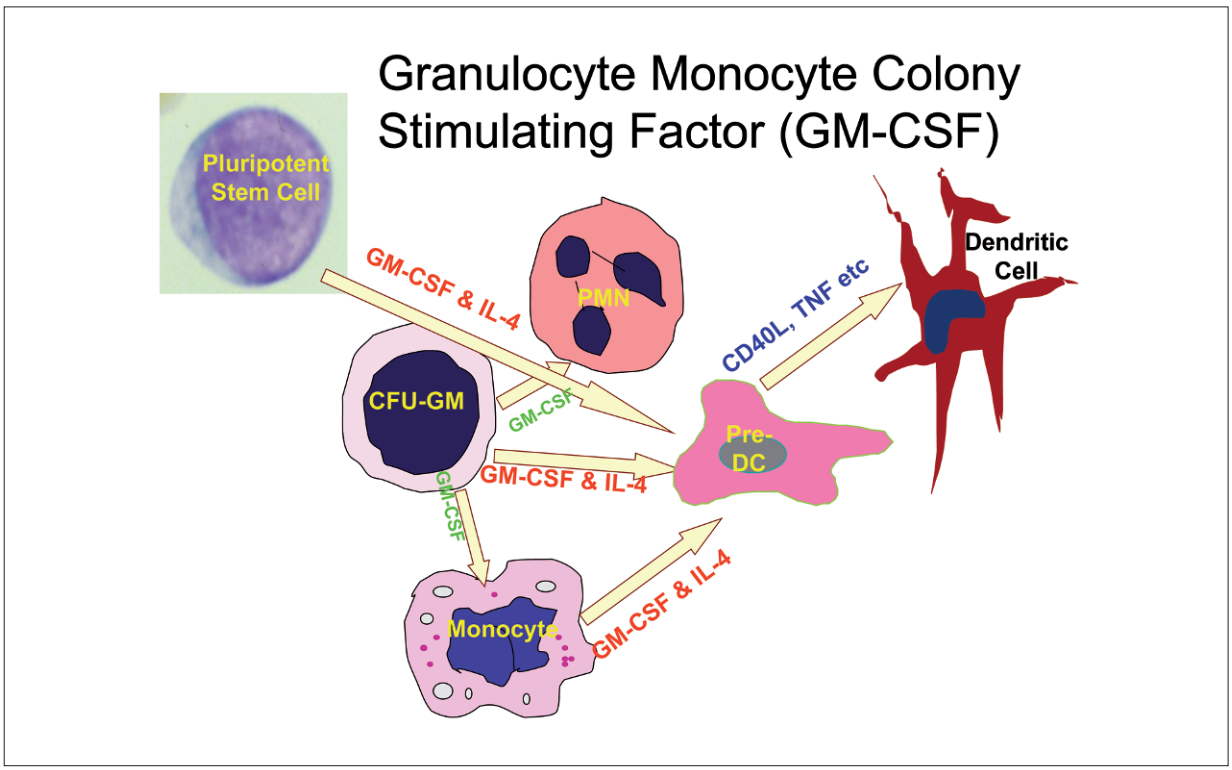

Figure 3. Maturation of precursors into DCs by granulocyte monocyte colony stimulating factor (GM-CSF)
GM-CSF and IL-4 can drive pluripotent stem cells, polymorphonuclear cells (PMNs), monocytes, and granulocyte-monocyte colony-forming unit (CFU-GM) genetic precursors into pre-DCs, which following activation can become mature activated DCs. CD40L, CD40 Ligand; TNF, tumor necrosis factor

GM-CSF effects on histiocytes

In addition to an effect on HEMATOPOIESIS, administration of GM-CSF can also affect MACROPHAGES and DCs. MACROPHAGES are functionally activated following stimulation with GM-CSF, and preclinical and clinical data suggests that GM-CSF can act as an adjuvant for VACCINES [86, 87]. DCs are Ag-presenting cells (APCs) that play a major role in the induction of primary and secondary T cell immune responses against cancer (see chapter A4). Because, GM-CSF is a mediator of proliferation, maturation and migration of DCs [88], it has been used to enhance the induction of Ag-specific CYTOTOXIC T cells. Due to the effect of GM-CSF alone or in combination with IL-4 on DCs, it has been used both *ex vivo* and *in vivo* to expand DCs for use as VACCINES [82, 89, 90], following transfection or priming with peptide Ags [82]. Further, preclinical studies have suggested that GM-CSF-transfected tumor cells can provide prophylactic and, in some instances, therapeutic anti-cancer activity [89–91]. Thus, GM-CSF has potential utility not only to address NEUTROPENIA, but also as a direct therapeutic agent within an adjuvant protocol or as a vaccine adjuvant.

Cancer patients have been shown to have defective macrophage and DC function [92, 93]. Preclinical studies have shown that MONOCYTES and tumor-associated histiocytes can be stimulated by GM-CSF to become CYTOTOXIC against tumor cells [94]. Because of the functional attributes of GM-CSF, a few clinical trials have studied GM-CSF either alone or in combination with IL-2 for the potential

to improve anti-cancer immunity. In a recent study, 48 cancer patients with surgically resected stage III or stage IV melanomas received GM-CSF s.c. for 14 days followed by monthly treatment for 1 year or until disease recurrence and the outcomes compared to matched historical controls. A median survival time of 37.5 months was observed with GM-CSF therapy *versus* 12.2 months for historical controls [95].

Engineered recombinant proteins

Several new RECOMBINANT BIOPHARMACEUTICALS have been engineered to improve their pharmacological properties. The primary strategy has been to improve the pharmacological half-life of proteins by PEGYLATION. This improves the BIOACTIVITY of a cytokine by decreasing systemic CLEARANCE, resulting in a reduced dosing frequency, improved PATIENT COMPLIANCE, and lowers costs. Many GROWTH FACTORS require daily administration, often in the clinic, and a weekly or bimonthly administration of a PEGYLATED protein is preferred. Indeed, there are a few reports suggesting improved outcomes for NEUTROPENIA, with PEGYLATED G-CSF, as compared to the parent protein [96], although there are concerns associated with splenomeglia and hyperleukocytosis [97] (Box 5). Similarly, genetic manipulation to increase the glycosylation of erythropoietin (EPO) has been shown to prolong its pharmacological half-life.

Several PEGYLATED IFNs have been studied. Whereas native IFNs have a relatively short half-life, typically 1–4 hours, PEGYLATION increases this to 24 hours or longer. As an example, IntronA (IFN-α2b) is typically administered three times a week, whereas PEGYLATED IFN is administered as a single weekly injection. Similarly, thrice-times weekly injections of Roferon (IFN-α2a) have been replaced by a single weekly injection of Pegasys, resulting in an increased EFFICACY for the treatment of hepatitis C. Most recently, PEGYLATED G-CSF has allowed a single injection of PEGYLATED G-CSF, replacing the requirement for daily or twice daily injections of G-CSF for 5 or more days.

Similar to PEGYLATION, the sequence of EPO was mutated to alter oligosaccharide sequences, resulting in improved pharmacokinetics. The sialic acid content of the carbohydrate component of glycoproteins has a significant effect on a protein's half life, which was exploited to create this novel EPO sequence (Aranesp). This resulted in increased sialic acid content from the addition of two extra glycosylation sites in the EPO backbone, and a longer half life. Additional strategies are being used to prolong

BOX 5. PEGYLATION

Colony-stimulating factors (CSFs) have significant bioactivity in the treatment or prevention of neutropenia. These cytokines were used originally as recombinant proteins, although recently pegylated G-CSF has shown improved pharmacological properties. In addition to significant clinical efficacy, these recombinant and pegylated growth factors have an economic impact within dose dense, standard of care, and myeloablative chemotherapy and stem cell transplantation. In general, growth factor administration is associated with a shorter duration of severe neutropenia; with antibiotic treatment, and in myeloablative therapy a reduced incidence of transfusions and days in the Intensive Care Unit are seen.

- Prolongs half-life and bioavailability.
- Protects against binding to monocyte and PMNs.
- Protects against enzyme degradation and Ab induction.
- Reduces number of injections required.
- Improved patient compliance.
- May result in increased bioactivity or a new profile of activity.

Table 2. Slow release variant drugs

Drug	Indication	Approval date
Aranesp (darbepoetin alfa)	Anemia associated with chronic kidney disease and chemotherapy induced anemia	June 01/July 02
IFN-α2a, Pegasys (PEG)	First-line treatment of chronic hepatitis C	October 02
Pegfilgrastim, Neulasta, G-CSF-PEG	Febrile neutropenia in patients receiving chemotherapy	January 02
PegIFN-α2b(PEG-Intron™)	Treatment of chronic hepatitis C in patients not previously treated with IFN-α	January 01

the half life of proteins, including nanoparticles, liposomes and poloxamer matrixes, which allow the slow release of a protein. These formulations provide not only a slow release, but also the potential for specific targeting to organs or tumors *via* modification of the formulation (Tab. 2).

Natural and synthetic biological response modifiers (BRMs)

Natural BRMs

The use of BRMs to treat human disease has its origins in the use of bacterial toxins to treat cancer by William B. Coley [98]. This form of therapy can act by multiple mechanisms as shown in Box 6. These early studies resulted in the clinical use of microbially derived substances such as BCG vaccine or picibanil, carbohydrates from plants or fungi such as krestin and LENTINAN, other products such as Biostim and Broncho-Vaxom, as well as thymic extracts (Tab. 3). However, there is considerable lot-to-lot variation in the purity of these compounds. In addition, due to the particulate nature of some of the BRMs, i.v. injections can result in pulmonary thrombosis and respiratory distress as well as in the development of granulomatous disease following dermal administration and scarification.

BCG

The most commonly used BRM for cancer therapy is BCG. It has been used systemically for the treatment

Box 6. Biological response modifiers

- Stop, control, or suppress processes that permit cancer growth, inflammatory or autoimmune process, including overcoming immunosuppressive processes.
- Induce processes that result in cancer cells being more recognizable and susceptible to destruction by the immune system.
- Boost the cytotoxic activity of effect cells, such as T cells, NK cells, and macrophages.
- Alter the growth patterns of cancer cells to promote a cellular behavior like that of healthy cells.
- Block or reverse the process that changes a normal cell or a precancerous cell into a cancerous cell.
- Enhance the body's ability to restore normal cells damaged or destroyed by treatment, such as chemotherapy or radiation.
- Prevent cancer cells from spreading (metastasizing) to other parts of the body.

Table 3. Natural BRMs

Agent	Chemical nature	Action	Clinical use
BCG (USA and EU)	Live mycobacteria	Macrophage activator	Bladder cancer
Biostim (EU)	Extract *Klebsiella penum.*	Macrophage activator	Chronic or recurrent infections
Krestin (PSK) (Jap)	Fungal polysaccharide	Macrophage activator	Gastric/other cancers
Lentinan (Jap)	Fungal polysaccharide	Macrophage activator	Gastric/other cancers
Picibanil (OK432) (Jap)	Extract *Strep. Pyogenes*	Macrophage activator	Gastric/other cancers
T-activin (Russia)	Thymic peptide extract	T cell stimulant	Cancer and infection
Thym-Uvocal (Germany)	Thymic peptide extract		Cancer and infection
Thymostimulin (EU)	Thymic peptide extract	T cell stimulant	Cancer and infection

of metastatic disease or adjuvant therapy, intralesionally (especially for cutaneous malignant melanoma), topically for superficial bladder cancer, and in combination with other immune modulators, tumor VACCINES and chemotherapy. Its use is FDA approved in the United States for intravesical administration for the treatment of superficial bladder cancer, residual disease and adjuvant activity [99]. Several randomized studies have shown a prolonged disease-free interval and time-to-progression in patients treated with intradermal and intravesical BCG as compared to controls [100]. Intravesical therapy with BCG, thiotepa, mitomycin, or doxorubicins is used in bladder cancer patients with multiple tumors, recurrent tumors or as a prophylactic approach in high-risk patients after transurethral resection (TUR). Treatment with BCG delays progression to muscle-invasive and/or metastatic disease, improves bladder preservation, and decreases the risk of death from bladder cancer [101, 102]. In one randomized study of patients with superficial bladder cancer, tumor recurrence was reduced with intravesical and percutaneous BCG administration as compared with controls [103]. Although intravesical BCG may not prolong OS for carcinoma *in situ*, it results in complete response rates of about 70%, decreasing the need for salvage cystectomy [104], and delays tumor recurrence and tumor progression [102, 105]. Results from a recent prospectively randomized trial suggested that maintenance BCG, when given to patients who are disease free after a 6-week induction course, may also improve survival [106]. In summary, meta-analyses have shown that BCG reduces recurrence and progression rates [107] and, in comparison to IFN-α within prospectively randomized trials, demonstrates similar EFFICACY [108]. It has been suggested that BCG plus IFN is a viable alternative in patients who fail intravesical BCG [109].

The mechanism by which BCG mediates its antitumor response is not known, but BCG treatment induces granulomatous INFLAMMATION [110] and elevates IL-2 levels in the urine of treated patients [111], suggesting that an augmented local immune response may be important. Clinical studies have shown that intravesical installation of BCG in patients with superficial bladder cancer results in a significant increase in IL-1β, IL-2, IL-6, TNF-α, IFN-γ and macrophage colony-stimulating factor (M-CSF) with a concomitant and significant increase in serum levels of IL-2 and IFN-α [112]. There appears to be a relationship between cytokine production and therapeutic EFFICACY, since a multivariant logistic analysis demonstrated that IL-2 induction was a discriminating parameter for remission in patients receiving BCG treatment for superficial bladder carcinoma [113]. This association of increased IL-2 levels was recently confirmed in a study of 20 patients with bladder cancer *in situ* [114].

Chemically defined BRMs

Synthetic BRMs

The use of NONSPECIFIC IMMUNOSTIMULANTS has also been extensively studied (Tab. 4). The microbially derived agents have in common widespread effects on the IMMUNE SYSTEM and side effects akin to infection (e.g., fever, malaise, myalgia, etc.). These agents can enhance nonspecific resistance to microbial or neoplastic challenge when administered prior to challenge (immunoprophylactic), but, rarely when administered following challenge (immunotherapeutic). This is an important distinction in that the primary objective for the oncologist is the treatment of pre-existent metastatic disease.

Levamisole

Following a long history of experimental use in many different cancers and diseases, LEVAMISOLE, a chemically defined, orally active immunostimulant, demonstrates significant therapeutic activity (meta-analysis) [115,116]. It was approved for the treatment of Duke's C colon cancer in combination with 5-fluorouracil (5-FU). LEVAMISOLE promotes T LYMPHOCYTE, macrophage and neutrophil function, suggesting multiple MOA. LEVAMISOLE stimulates T cell function *in vivo*, particularly in immunodeficient individuals, presumably through the action of its sulfur moiety. One study with LEVAMISOLE demonstrated a significant increase in the frequency of PB mononuclear cells expressing the NK cell Ag CD16 at all dose levels, although lower toxicity was observed at the lower doses of LEVAMISOLE [117]. The authors suggested that short-term LEVAMISOLE administration was only minimally immunomodulatory, and that chronic administration at low doses may be better tolerated and provide similar levels of immune modulation to that observed with higher doses [117]. It is relatively non-toxic (flu-like symptoms, gastrointestinal upset, metallic taste, skin rash and Antabuse reaction), but can produce an agranulocytosis, particularly in human leukocyte Ag (HLA) B-27$^+$ patients with RHEUMATOID ARTHRITIS where its use has been discontinued. The adjuvant therapeutic activity of LEVAMISOLE has been questioned in recent years. In one phase III trial, comparing 5-FU with leucovorin to 5-FU with LEVAMISOLE, it was found that the 5-FU and LEVAMISOLE significantly prolonged DFS and OS in patients with type III colon cancer who had undergone curative resection relative to adjuvant therapy with LEVAMISOLE [118]. In a recent report from the Norwegian Gastrointestinal Cancer Group [119], it was found in a randomized phase III study of adjuvant chemotherapy with 5-FU and LEVAMISOLE for the treatment of stage II and III colon and rectum cancer that there was no significant survival difference between treatment with or without LEVAMISOLE. However, there was a subgroup of colon cancer patients with stage III disease who had a statistically significant difference in their DFS and cancer-specific survival in favor of adjuvant chemotherapy. It was concluded that colon cancer patients with lymph node metastases benefit from adjuvant chemotherapy with 5-FU in combination with LEVAMISOLE.

Amino bisphosphonates

Bone is the most frequent site of metastasis by breast and prostate tumors, often resulting in loss of bone mineral density (BMD). In addition, treatment with chemotherapeutics and aromatase inhibitors, can result in reduced BMD. Bisphosphonates, SYNTHETIC analogues of naturally occurring pyrophosphate compounds, are the standard treatment for BMD, in addition to their potential to reduce bone pain and bone metastases. Randomized trials have shown that bisphosphonates can reduce the risk of bone fracture, cord compression, hypercalcemia and need for palliative radiation [120–123]. Amino bisphosphonates also have direct effects on tumor cells, in addition, to their inhibition of osteoclast bone resorption [124]. They have been suggested to have anti-angiogenic activity [125], in part due to the inhibition of osteoblastic cell secretion of VEGF [126]. Santini reported that the oral bisphosphonate pamidronate reduced VEGF levels in cancer patients with bone metastases, and that pamidronate and zelondratic acid (ZA) reduced bFGF- and VEGF-induced proliferation of vascular tissue in mice [126,127]. In addition, ZA has been reported to suppress myelopoiesis, including myeloid-derived suppressor cells (MDSC), helping to overturn the tumor-induced immune suppression

Table 4. Chemically defined BRMs

Agent	Chemical nature	Action	Clinical use
Adenosine	Purine nucleotide	Innate immune stimulant	Asthma, sepsis, cancer
Aranesp	Glycosylated EPO	Erythropoietic stimulating agent	Anemia
Azathiopine Imuran	Purine antimetabolite	Immunosuppressant	Graft *versus* host disease, allograft
Bestatin (Jap)	Dipeptide	Macrophage and T cell stimulant	Acute myelosis leukemia
Celebrex	Celecoxib	COX-2 in inhibitor	Anti-inflammatory and familial adenomatous polyposis.
Cyclosporine Sandimmune®	Cyclic undecapeptide which is a metabolite of soil fungus	Immunosuppressant	Graft *versus* host disease, allografts, rheumatoid arthritis, psoriasis
Cytosine-phosphate-guanine	Nucleotide	Binding to TLR9 and DC activity	Vaccine adjuvant
Deoxyspergualin	Peptide fermentation product of bacillus laterosporus	Immunosuppressant	Acute renal rejection
Everolimus (Certican)	TOR kinase inhibitor	Immunosuppressant	Cardiac transplant
Isoprinosine (Eur)	Inosine:salt complex	T cell stimulant	Infection
Lenalidomide	Thalidomide analogue	T cell stimulant	Myelodysplastic syndrome with 5q deletion; multiple myelomas
Levamisole (USA)	Phenylimidothiazole	T cell stimulant	Cancer
Mifamurtide	Muramyl tripeptide phosphatidyl-ethanolamine	Macrophage stimulating drug	Osteosarcomas
Mycophenolate moeftil (MMF) Celleept®	2-morpholine ethylester of mycophenolic acid	Immunosuppressant	
Rapamycin Rapamune® (sirolimus)	Metabolite of *Streptomyces hygroscopicus*	Immunosuppressant	Solid organ transplantation, graft *versus* host disease
Revlimid (lenulidomide)	Thalidomide analogue	Suppresses TNF and adhesion molecule expression	Low and intermediate risk MDS
Romurtide (Jap)	18 lys MDP	Macrophage stimulant	Bone marrow recovery
Sunitinib (Sutent)	Tyrosine kinase inhibitor	Inhibitor of MDSCs	Renal cell carcinoma

Table 4. *(continued)*

Agent	Chemical nature	Action	Clinical use
Tacrolimus, FK505, Prograf®	Macrolide lactone which is a fermentation product of *Streptomyces*	Immunosuppressant	Eczema, solid organ transplantation, graft *versus* host disease
Thalidomide (USA)	α-(*N*-phthalimido)glutarimide	Suppresses TNF and adhesion molecule expression	Erythema nodosum leprosum
Thymopentin TP-5 (Italy and Germany)	Pentapeptide	T cell stimulant	Rheumatoid arthritis infection and cancer
Zelondratic acid (ZA)	Bisphosphonate	Inhibitor of VEGF, MMP-9 and MDSCs	Bone mineral density, bone metastases, and Paget's disease

and stimulate antitumor immune responses [128]. The growth of tumors and the administration of chemotherapeutic drugs have been shown to increase the frequency of myeloid cells, including cells that can suppress T cell number and function [129–131]. These cells, identified in mice as MDSC (Fig. 4) can suppress T cell function [132, 133], and are also found in cancer patients [134, 135]. In humans, the phenotype of MDSCs has been reported to be DR^-, Lin^-, $CD11b^+$ and $CD33^+$ [136–139]. Several strategies have been used, in rodent models and clinically, to reduce their function and decrease their accumulation, resulting in an improved T cell responses and apparent therapeutic activity [140–149].

Sunitinib

SU11248 or Sutent® is another drug that has been identified with the potential to regulate MDSCs. It was developed as an oral, multitargeted RECEPTOR tyrosine kinase (RTK) inhibitor with antitumor and anti-angiogenic activity. It targets platelet-derived growth factor RECEPTOR (PDGFR), VEGF RECEPTOR (VEGFR), KIT, and Flt3. Thus, sunitinib has antitumor activity by dint of inhibition of tumor cell proliferation, survival, and vascularization *via* an effect on endothelial cells [150]. In addition, it has been suggested to have therapeutic activity *via* the inhibition of MDSC expansion [5, 151–154]. Preclinical studies using autochthonous rodent tumor models and human tumor XENOGRAFTS have demonstrated inhibition of TARGET RTKs and concurrent tumor growth effects with sunitinib [151, 155, 156] (Box 7). Sunitinib monotherapy has also been shown to induce the regression of mammary tumors in MMTV-v-Ha-ras transgenic mice (82% regression) and 7,12-dimethylbenz(a) anthracene-induced mammary tumors in rats [156]. Sunitinib is currently the standard of care for renal cell carcinoma patients who have a high expression level of VEGF and PDGF [156, 157]. It is also FDA-approved for the treatment of gastrointestinal stromal tumors (GIST) in patients who are intolerant of, or unresponsive to imatinib [158]. Sunitinib can also reduce Flt3-driven phosphorylation, induce APOPTOSIS *in vitro* and inhibit FLT3-induced VEGF production [159]. This is an important attribute of the activity of sunitinib as LIGAND-mediated activation of the Flt3 RECEPTOR is important for normal proliferation of primitive hematopoietic cells. Indeed, RTK inhibitors with Flt3 AFFINITY have been shown to suppress plasmacytoid DC (pDC) and DC development in Flt3L-supplemented mouse BM cell cultures.

Muramyl dipeptides – Synthetic natural BRMs

One of the largest and best studied classes of SYNTHETIC agents is that based on MURAMYL DIPEPTIDES (MDP). MDP was discovered as the minimally active substitute for intact BCG in Freund's adjuvant [160–162]. Unfortunately, as with many of the polypeptides with low molecular weights, MDP has a short serum half-life and requires frequent administration at high

Box 7. Sunitinib

Sunitinib is a multitargeted tyrosine kinase inhibitor that can selectively inhibit vascular endothelial growth factor receptor (VEGFR) 1, VEGFR2, and VEGFR3; platelet-derived growth factor receptor (PDGFR)α and PDGFβ; Flt3-L and stem cell factor [255, 256]. Clinical studies have explored the anti-angiogenic activity of sunitinib [255, 256]. *In vivo*, sunitinib treatment can reduce tumor microvessel density, inhibit neovascularization, and prevent metastases [257]. It has been approved for use as an anti-angiogenic drug in patients with gastrointestinal stromal tumors (GIST) and renal cell carcinoma (RCC). In the latter disease, it has become frontline therapy [258, 259]. Although sunitinib has been explored in the clinic mainly for its anti-angiogenesis effects, it has also been suggested to induce marked tumor cell necrosis, not associated with an effect on tumor vasculature [259]. Recent preclinical [260] and clinical studies have also shown that sunitinib treatment results in reduced numbers of myeloid-derived suppressor cells (MDSCs) [152, 153, 261], improved Th1 response, and diminished Treg cells in RCC patients. These studies suggest that mechanisms mediating sunitinib's therapeutic activity may extend beyond its anti-angiogenic activity.

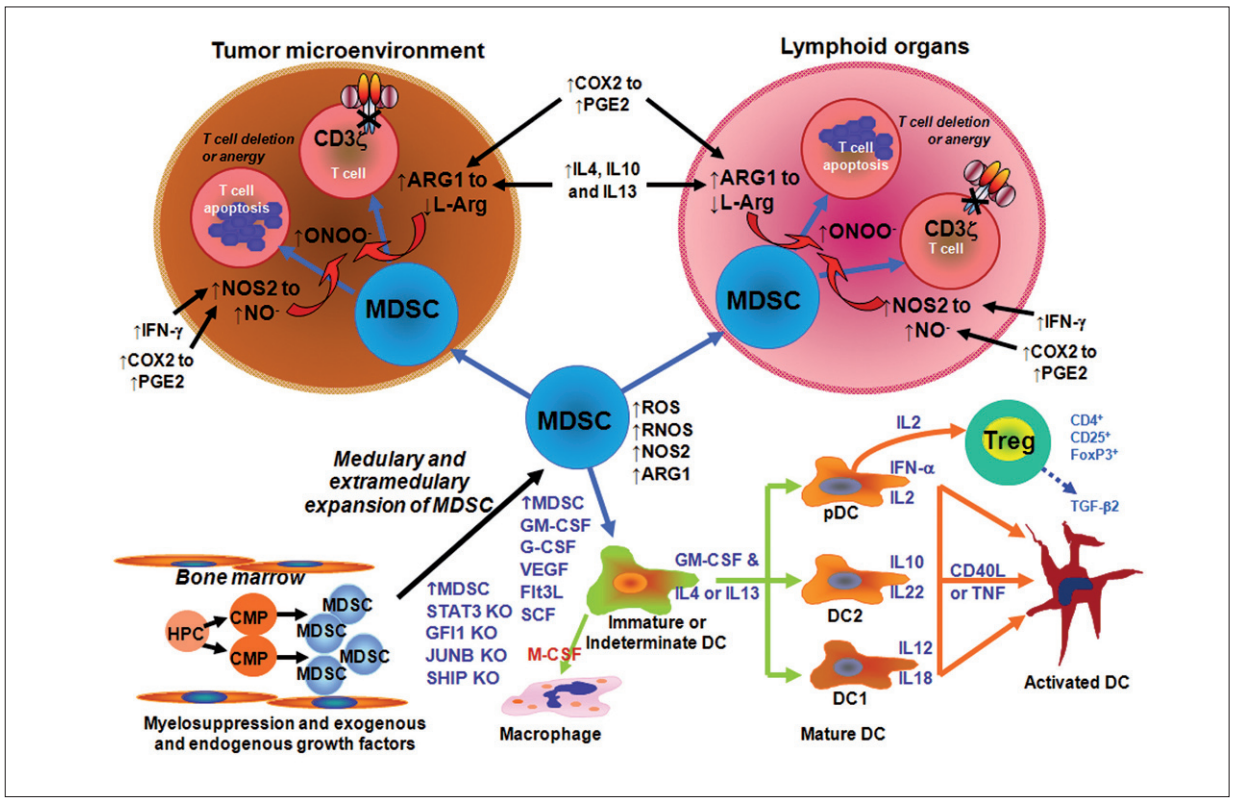

FIGURE 4. EXPANSION AND REGULATION OF MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs)

Hematopoietic progenitor cells (HPCs) proliferate, differentiate, and commit to various hematopoietic lineages, including committed myeloid progenitors (CMPs). Under conditions of myelosuppression or exogenous administration of growth factors, significant increases in MDSCs occur in the peripheral blood (PB) and spleen, and infiltrate tumors. MDSCs, during expansion, are also mobilized into the circulation, lymphoid organs, and tumor microenvironments. The expansion of MDSCs is associated with increased levels of growth factors or VEGF. In addition to proliferation, MDSCs can also be activated, secreting high levels of ARG1 and NOS2. ARG1 decreases l-arginine levels, resulting in a translational blockade of the CD3ζ chain. This is T cell suppressive, affecting multiple pathways, including the kinases, GCN2, and mTOR. In addition, high levels of NOS2 and NO are induced, which nitrosylate cystine residues in target proteins and affect the production of cyclic GMP. This affects IL-2 receptor signaling by blocking the phosphorylation of signal-inducing molecules coupled to IL-2R and altering the stability of IL-2 mRNA. Up-regulation of both enzymes, in addition to affecting the two pathways described above, can increase the production of other ROS and RNOS, including O_2^-, NOO^-, and H_2O_2. This occurs either by nitrating tyrosine residues or by controlling BCL-2 and CD95L expression, resulting in T cell apoptosis. Signaling elements that have been shown to regulate this process include STAT3 [262–264], GFL-1 [265], JunB [266], and SHIP [267]. Furthermore, MDSCs can differentiate into DCs, which can be matured by M-CSF into macrophages or into DCs by GM-CSF and IL-4 or IL-13. This includes myeloid DCs, which are immunosuppressive via the production of IL-10 and IL-21. These may differentiate into lymphoid or myeloid DCs with the production of immune-augmenting levels of IL-12 and IL-18. Alternatively plasmacytoid DCs (pDCs) may be formed, which have important roles in the response to viral infections via the production of IL-2 and interferon, the high levels of IL-2 increasing the frequency of Treg cells. All of these DC types can be activated by a variety of cytokines, including CD40L and TNF-α, to express increased levels of costimulatory molecules.

doses to be active. In addition, agents such as MDP are strongly pyrogenic, presumably in association with their ability to induce IL-1. MDP analogues have also been incorporated into multilamellar vesicles for higher stability and to facilitate monocytic PHAGOCYTOSIS.

The first MDP that was approved for clinical use was romurtide (Japan), which induces BM recovery following cancer chemotherapy [163] *via* growth factor induction. Its mechanism of action (MOA) is the activation of MACROPHAGES to secrete CSFs, IL-1 and TNF, resulting in the expansion of marrow precursors and subsequent commitment and differentiation into mature GRANULOCYTES and MONOCYTES. Therefore, the period of granulocytopenia and the risk of secondary infections are reduced, allowing more frequent and/or intense chemotherapy. MURABUTIDE (Tab. 4), an orally active form of MDP that does not induce fevers, is currently in clinical trials with cancer and infectious disease (France). In order to further stabilize the incorporation of MDP into multilamellar vesicles, lipophilic analogs of MDP such as muramyl tripeptide phosphatidylethanolamide [MTP-PE (mifamurtide)] have been developed. MTP-PE has shown significant therapeutic activity in pediatric patients with osteosarcoma [160, 162, 164] and was recently approved in Europe for the treatment of non-metastatic osteocarcoma following surgical resection. Preclinically, MTP-PE has also shown protection of the mucosal epithelium from cytoreduction therapy [160, 161, 165]. The MDPs are also potent adjuvants, either alone or in oil emulsion and are under consideration for use as adjuvants with HIV peptide VACCINES.

Bestatin – Engineered synthetic natural BRMs

Bestatin (ubenimex) is a potent inhibitor of aminopeptidase N and aminopeptidase B [166], which was isolated from a culture filtrate of *Streptomyces olivoreticuli* during the search for specific inhibitors of enzymes present on the membrane of eukaryotic cells [167]. Inhibitors of aminopeptidase activity are associated with macrophage activation and differentiation, and Bestatin has shown significant therapeutic effects in several clinical trials [168]. In one multi-institutional study, patients with acute nonlymphocytic leukemia (ANLL) were randomized to receive either Bestatin or control [169] orally after completion of induction and consolidation therapy, and concomitant with maintenance chemotherapy. Remission duration was prolonged in the Bestatin group, although this difference did not reach statistical significance. However, OS was prolonged in the Bestatin group. Recently, a confirmatory phase III trial in ANLL was reported that extended the observation to a significant prolongation of remission [170]. Bestatin has also shown adjuvant activity when administered to acute leukemia and CML patients who did not develop graft-*versus*-host disease (GVHD) within 30 days following BMT [171]. Bestatin-treated acute leukemia patients had an increased incidence of chronic low-grade GVHD compared with the control arm and a lower relapse rate. Recently, a phase III study of resected stage 1 squamous cell lung cancer patients treated orally with either Bestatin or placebo daily for 2 years revealed that 5-year cancer-free survival was significantly greater in the Bestatin group (71%) as compared to the placebo group (62%). OS was also significantly improved, as was cancer-free survival [172]. Recent studies in patients with non-small cell lung cancer (NSCLCs) suggest that it also has anti-angiogenic activity [173].

Oligonucleotides as natural/synthetic BRMs

BACTERIAL EXTRACTS can activate both innate and ADAPTIVE IMMUNITY. The molecular RECEPTORS that regulate how the INNATE IMMUNE SYSTEM detects infectious agents and distinguishes different classes of pathogens have recently been elucidated. The IMMUNE SYSTEM uses 'PATTERN RECOGNITION RECEPTORS' that are expressed on certain innate immune cells to trigger cellular activation when they recognize conserved microbial-specific molecules [174, 175]. These molecules, originally thought of as nonspecific immune activators as discussed above, are now known to be specifically recognized by RECEPTORS that are expressed in a cell-specific and compartmentalized manner (see chapter A6). The best-characterized family of PATTERN RECOGNITION RECEPTORS is the Toll-like RECEPTOR (TLR) family. One of these, TLR9 is expressed in the endosomal compartment of

plasmacytoid DCs and B cells [176], and is essential for the recognition of viral and intracellular bacterial DNA [177]. Based on the identification of these specific ligands, IMMUNOTHERAPY has begun to grow beyond the nonspecific effects of whole BACTERIAL EXTRACTS, using SYNTHETIC TLR ligands (TLRLs).

One example of such SYNTHETIC immunomodulatory molecules are the short oligodeoxynucleotides (ODNs) that mimic the innate immune response to microbial DNA. These contain one or more cytosine-phosphate-guanine (CpG) dinucleotide-containing motifs with unmethylated cytosine residues, which are recognized by TLR9. The immune effects of CpG ODNs occur in two stages: an early stage of innate immune activation and a later stage of enhanced ADAPTIVE IMMUNITY (Fig. 5). Within minutes of the exposure of B cells or pDCs to CpG, the expression of COSTIMULATORY MOLECULES, resistance to APOPTOSIS, up-regulation of the chemokine RECEPTOR CCR7 (associated with trafficking to the T cell zone of the lymph nodes) and secretion of Th1-promoting CHEMOKINES and CYTOKINES, such as macrophage inflammatory protein-1 (IP10) and other IFN-inducible genes [178], are observed. pDCs secrete IFN-α and mature into highly effective APCs [179]. The CpG-induced secretion of IFN-α, TNF-α, and other CYTOKINES and CHEMOKINES induce, within hours, secondary effects, including NK cell activation and enhanced expression of Fc RECEPTORS, resulting in increased ADCC. This innate immune activation and pDC maturation into myeloid DCs is followed by the induction of adaptive immune responses. B cells are strongly costimulated if they bind specific Ag at the same time as CpG, which selectively enhances the development of Ag-specific Abs [180]. CpG binding also activates B cells to proliferate, secrete IL-6, and differentiate into plasma cells [181]. The CpG-enhanced APC function occurs *via* the up-regulated expression of

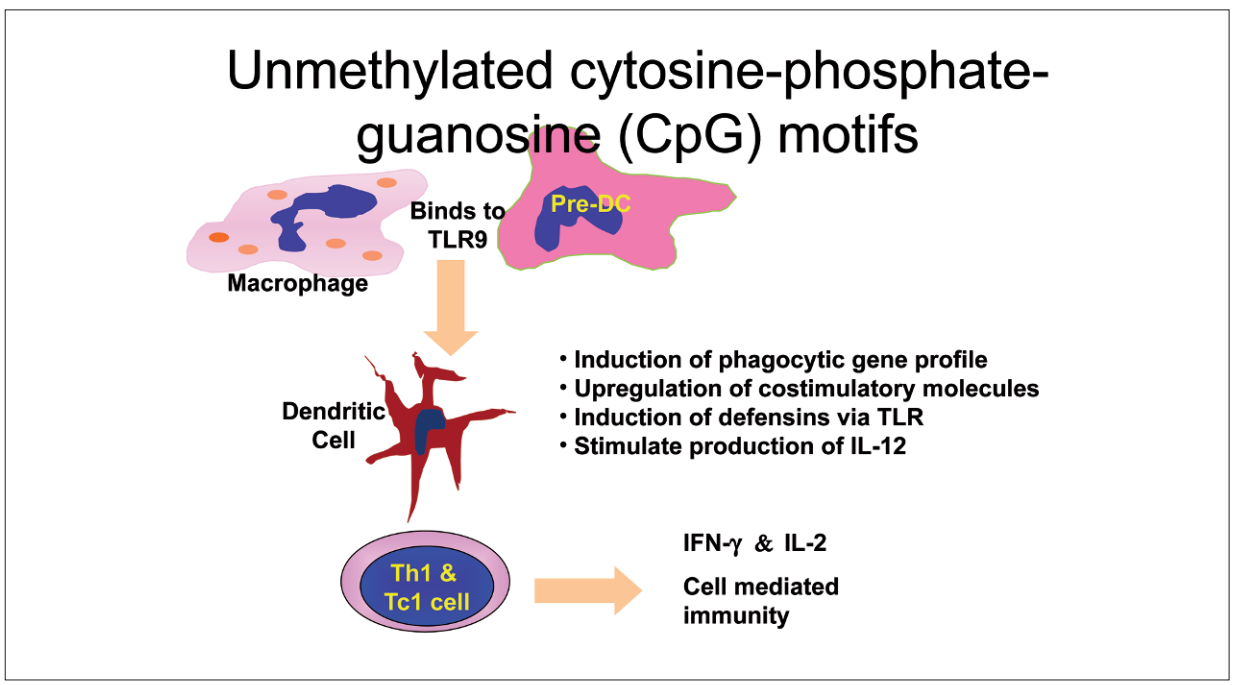

FIGURE 5. REGULATION OF INNATE AND ADAPTIVE IMMUNITY BY CYTOSINE-PHOSPHATE-GUANINE (CPG) MOTIFS
The presence or injection of unmethylated CpG motifs results in their binding to Toll-like receptor 9 (TRL9), leading to the activation and maturation of DCs, which can interact with Th1 or Tc1 cells and up-regulate cell-mediated immunity. In addition, CpG can induce phagocytosis, up-regulation of costimulatory molecules, defensins, and IL-12.

COSTIMULATORY MOLECULES, including CD40, CD80, and CD86 [182]. In mixed cell populations, a cascade of secondary responses, including activation of MACROPHAGES and NK cells and the induction of IFN-γ by Th1 cells also occurs [182–184]. The efficient activation of APCs and induction of IL-12, IL-18, IFN-α, and IFN-γ is directly associated with the ability of ODNs to induce Th1 polarization, adjuvant activity [185] inhibition of Th2 responses [186] and stimulate CD8 T cell responses [183].

CpG ODNs also appear to be effective as vaccine adjuvants to enhance adaptive Th1 cellular immune responses [187–189]. In mice, CpG ODNs can trigger strong Th1 responses [188, 189], enhancing the number and function of tumor-specific CTLs and IFN-γ-secreting T cells [190, 191]. CpG ODNs also enhance therapeutic responses to VACCINES, including DC VACCINES [192], proteins, irradiated cells transduced with GM-CSF [193, 194], and long peptide VACCINES [190]. This has resulted in therapeutic VACCINES in mouse tumor models where no other approach has shown comparable EFFICACY, even with 1-cm established tumors [192, 195]. Even without a vaccine, CpG ODNs can induce $CD8^+$ T cell-mediated regression of established tumors with durable memory responses [196]. By the stimulation of INNATE IMMUNITY, TLR activation by CPG analogues has been shown to enhance Ag-specific humoral and cellular immune responses, such that CPG ODN have seen increasing utility as vaccine adjuvants [197, 198]. In one study, melanoma patients, who were randomized to preoperative administration of either PF-3512676 or saline, developed IFN-γ^+ $CD8^+$ T cells, demonstrated by ELISpot analysis from sentinel lymph nodes (SLNs) and PB, against melanoma-associated Ags (MAAs). In this study, there were no $CD8^+$ T cell responses to MAA in the 11 members of the saline group, compared to responses in 5 of 10 patients who received PF-3512676, which was significantly higher. The $CD8^+$ T cell response was also found to correlate with the activation of pDCs in the SLNs [199]. In another clinical study, administration of CpG ODN PF-3512676 in combination with taxane/platinum chemotherapy to chemotherapy-naïve patients with stage IIIb/IV NSCLC [200] resulted in a significant, higher overall response rate (38%) compared to patients randomized to standard chemotherapy (19%). The median overall survival was 6.8 months in the chemotherapy arm, *versus* 12.3 months in the combination arm. In contrast, a phase III study combining PF-3512676 with Paclitaxel/Carboplatin or gemcitabine/cisplatin *versus* chemotherapy alone as first-line treatment for patients with advanced NSCLC [201] resulted in no improvement in OS or progression-free survival when PF-3512676 was added to standard chemotherapy.

Combination immunotherapy and cellular therapy

Stem cell transplantation: Demonstration of T cell therapeutic activity

Because CYTOKINES have unique MOAs, they are ideal candidates for COMBINATION THERAPY with chemotherapeutic agents (Box 8). However, increased knowledge and consideration of the potential interactions are necessary for successful clinical use. The use of HIGH-DOSE CHEMOTHERAPY (HDT), which is myeloablative (BM destructive) and requires stem cell rescue, provides the ultimate in cytoreductive therapy. Further, SCT provides one of the few statistically supported demonstrations of clinical therapeutic EFFICACY by T cells based on the survival of patients receiving an allogeneic *versus* an autologous transplant [202]. Thus, strategies to up-regulate T cell function after autologous SCT provides one focus for cytokine therapy. This is important as the return of immunological function in transplant patients is slow and accompanied by depressed numbers of $CD4^+$ T cells, a low CD4/CD8 T cell ratio, and suppressed T cell responses [160, 203].

The role of T cells in controlling neoplastic disease is described as a graft *versus* tumor (GVT) response. This role of T cells in treating neoplasia is supported by the significantly higher risk of relapse in patients receiving an allogeneic SCT, which has been T CELL DEPLETED (TCD) or who receive CICLOSPORIN (CsA) to prevent GVHD [204]. However, GVHD also has unfavorable effects on transplant-related morbidity and mortality. In first remission, the decreased relapse rate with acute and/or chronic

Box 8. Combination chemo-immunotherapy (biochemotherapy)

- Immunotherapy is most active against minimal residual disease.
- Immunotherapy utilizes mechanisms of action that differ from chemotherapy and as such has a different resistance profile.
- Chemotherapy generally results in a reduction in the extent of neoplastic disease, but frequently does not remove all residual tumor cells.
- Tumor resistance to chemotherapy frequently occurs such that high doses are often needed to assure maximal effect.
- In part, because immunotherapy utilizes different mechanisms of action, additional efficacy can be expected.
- Chemotherapy and other cytoreductive therapies are generally toxic, while immunotherapy generally is minimally toxic.
- Chemotherapy can reduce the number of host effector cells capable of responding to a tumor and limit the extent of immunotherapy.
- In contrast, chemotherapy, often in a dose-dependent manner, can be immune augmenting by reducing immune suppressor cells.
- Based on the above considerations, significant insight into the pharmacology and toxicology of the therapeutic agents is needed to successfully combine chemotherapy and immunotherapy.

GVHD is more than offset by the increased risk of death from other causes. Consequently, patients with GVHD have a lower risk of treatment failure, but an increased risk of morbidity due to GVHD.

TCD SCT markedly reduces the incidence of severe GVHD. However, as discussed above, T cell depletion is also associated with an increased rate of severe, and often, fatal infections, a higher incidence of graft rejection, and an increased risk of disease recurrence. The increased risk of infectious complications is associated with the slow recovery of $CD4^+$ and $CD8^+$ T cells that occurs following SCT, as the initial T cell recovery that occurs with an unmanipulated stem cell product is associated with the T cells transplanted with the STEM CELLS [202]. Similarly, the increased graft failure that is observed following transplantation with a TCD product likely reflects the contribution that infused T cells make towards the eradication of residual host T cells following the transplant preparative regimen. Due to the increased incidence of infections and relapse, donor leukocyte infusions (DLI) may be used to reduce the incidence of graft loss, disease relapse and secondary infections [205]. However, DLI is also associated with an increased risk of GVHD, and thus, alternatives to TCD and DLI, such as strategies that can induce Ag-specific TOLERANCE shortly after allogeneic SCT, are appealing as they might prevent GVHD without resulting in a requirement for post-graft IMMUNOSUPPRESSION.

SCT and immunotherapy

Adjuvant studies in patients receiving HDT and SCT include a focus on IMMUNOTHERAPY. The dose-intense preparative regimens, commonly referred to as HDT, are administered before transplantation and a number of cytokine- and/or vaccine-associated protocols are given following transplant with a TCD product or intact product in an attempt to improve immunological function, particularly those directed against tumor cells. One THERAPEUTIC STRATEGY is the use of VACCINES capable of inducing Ag-specific effector T cells. In addition, T cells from the donor may be stimulated *ex vivo*, expanded and then reinfused. Strategies have also focused on the initiation of CTL response to viruses, which can reduce the incidence of treatment-related Epstein-Barr virus (EBV)-associated lymphomas or infections such as cytomegalovirus (CMV) [206, 207].

SCT combination therapy with IL-2

One approach to improving survival of cancer patients has been the use of IL-2 IMMUNOTHERAPY following HDT and SCT to induce an autologous GVT response. Based on this strategy, studies using IL-2 alone following SCT have shown an increase in NK cell phenotype and function [160, 208–210]. In one study [211] with 18 evaluable patients, three responses were observed. In another study, IL-2 was infused for a median of 85 days following both autologous and allogeneic SCT [210]. Toxicity was minimal, and the treatment was undertaken in the outpatient setting *via* a Hickman catheter. In this study, no patient developed any signs of GVHD, hypertension or pulmonary capillary-leak syndrome. Despite the administration of low-dose IL-2, significant immunological changes were noted with a 5–40-fold increase in NK cell number. In addition, there was a significant augmentation of *ex vivo* CYTOTOXICITY against K-562 and colon tumor targets. In a similar study, it was shown that following continuous infusion of IL-2 in patients receiving autologous SCT, the $CD3^+$ and $CD16^+$ cells secreted increased levels of IFN-γ and TNF following *in vitro* culture, and there was a significant increase in serum levels of IFN-γ, but not TNF [210]. Recently, post-transplantation IL-2 administration has been extended to include the use of IL-2 or IL-2 and G-CSF for the mobilization of STEM CELLS [212, 213]. The objective of using IL-2 in this context is to mobilize T cells or change the population of T cells to those that may exert improved antitumor activity as well as the potential to reduce secondary infections.

SCT combination therapy with IFN-α

Similar post-transplantation strategies with IFN-α have been undertaken with the suggestion of a reduced risk of relapse and an increase in myelosuppression [214, 215]. In an early study of the prophylactic use of IFN-α following allogeneic BMT, the Seattle group [215] found that adjuvant treatment with IFN-α had no effect on the probability or severity of CMV infections or GVHD in acute lymphocytic leukemia (ALL) patients who were in remission at the time of transplantation. In this large study, there was a significant reduction in the probability of relapse in the IFN-α recipients ($p=0.004$) as compared to transplant patients who did not receive IFN-α, although survival rates did not differ between the two groups. It was suggested that the administration of IFN-α following transplantation reduced the risks of relapse, but did not affect CMV infection, perhaps because IFN-α was not initiated until a median of 18 days following transplantation and was not administered chronically.

SCT combination therapy with CSA

Recently, Ratanatharathorn et al. [216] undertook studies to induce a GVT reaction by administration of both CSA and IFN-α to augment GVHD in autologous transplant patients. Of the 22 patients enrolled, 17 were considered evaluable. Thirteen of the patients who received Hu-IFN-α2a developed GVHD, regardless of whether they received CSA, whereas only 2 of the 4 patients who received CSA alone developed detectable GVHD. Patients receiving 1×10^6 U/day Hu-IFN-α2a concomitant with CSA showed a trend towards increased severity of clinical GVHD as compared to patients receiving CSA alone ($p=0.06$). They concluded that IFN-α administration can be safely started on day 0 of autologous BMT and can induce autologous GVHD as a single agent with the potential to improve therapy.

In similar studies, Kennedy et al. [217] treated women with advanced breast cancer with a combined therapy of CSA for 28 days using a dose of 0.025 mg/m^2 of s.c. IFN-γ every other day on days 7–28 after HDT and autologous BMT. They observed that autologous GVHD developed in 56% of the patients, an incidence comparable to that previously observed with CSA alone. The severity of GVHD was greater with CSA plus IFN-γ than with CSA alone, as 16 patients required corticosteroid therapy for dermatological GVHD. Recently, IFN-γ therapy was administered following DLI on a patient who received a matched sibling graft to treat first chronic phase CML [218].

SCT combination therapy with IL-12

Adjuvant IMMUNOTHERAPY studies following autologous peripheral SCT (PSCT) have used patients with

hematological malignancies, and dose-escalation IL-12 studies following myeloid recovery. IL-12 is a heterodimeric cytokine that can stimulate both innate and ADAPTIVE IMMUNITY [219]. It is produced predominately by DCs, supports the proliferation of activated T cells and promotes the differentiation of T cells into Th1 and EFFECTOR CELLS [220, 221]. Further, IL-12 activates and augments the cytolytic activity of NK cells [222] and stimulates IFN-γ production by both NK and T cells [223]. In preclinical tumor models, IL-12 therapy has been shown to induce regression of established primary tumors, inhibit the formation of tumor metastasis and to prolong the survival of tumor-bearing animals [224, 225]. Objective, complete and partial responses have been seen in patients with advanced solid tumors who had received IL-12 in phase I clinical trials [226]. Based on these results, a dose-finding study was established [227] in which IL-12 was administered as a single bolus i.v. injection, followed 2 weeks later with daily injections for 5 consecutive days with cycles repeated every 3 weeks. A transient NEUTROPENIA and thrombocytopenia was seen at all doses. Biological activities included an increase in serum IFN-γ levels, and effects on the numbers of T cells, B cells and NK cell were observed at all three dose levels. A slight NEUTROPENIA occurred with IL-12 administration and a significant decrease in total CD4 cells. In contrast, there was an increase in CD8 and NK cells, but no effect on B cells following the administration of IL-12. Serum IFN-γ levels peaked from 12 to 24 hours following administration at 100 and 250 ng/kg. Based on these studies, it appears that an appropriate dose of IL-12 has been identified for future studies to examine EFFICACY following PSCT.

SCT and vaccine or adoptive cellular therapy

Immunization and adoptive CELLULAR THERAPY strategies TARGET the activation and expansion of tumor-reactive T cell populations in hosts with an intact IMMUNE SYSTEM. However, immunity, within cancer patients, is dysregulated in association with tumor-induced suppression and iatrogenic manipulation. As a consequence, immunosuppressive CYTOKINES and cells are increased, potentially limiting the effectiveness of vaccine-induced tumor-specific T cells and/or expansion of adoptively transferred T cells [228]. One approach to address this challenge has been to induce lymphopenia or reduce/oblate cellular suppressors in patients, allowing residual host or adoptively transferred, naïve or Ag-specific T cells to undergo homeostasis-driven proliferation and activation to restore memory and effector T cell compartments. This approach has several potential advantages including the elimination of inhibitory immune cells, such as Tregs, allowing lymphoid reconstitution to overcome inherent defects in T cell signaling and to improve Ag presentation by APCs *via* an up-regulation of costimulatory factors [229]. Prior studies have reported that immunomodulatory doses of cyclophosphamide can enhance vaccine-induced anti-tumor immune responses by inhibiting suppressor T cell activity [1, 230]. The administration of fludarabine, which is lymphodepleting, but minimally myelodepleting, provides another approach either as a single agent or in combination with cyclophosphamide. It has been used as a non-myeloablative pre-operative regime with adoptive transfer of tumor-reactive T cells in patients with metastatic melanoma [231]. Lymphopenia also results in heightened secretion and availability of immune stimulatory CYTOKINES including IL-7 and IL-15 that can enhance T cell function [232]. Some studies have shown enhanced T cell trafficking into tumors during and following the induction of lymphopenia [232–234]. In association with the expansion of T cell trafficking into tumors, there is an enhanced intratumoural proliferation of EFFECTOR CELLS following vaccination after non-myeloablative therapy and vaccination [235]. Thus, it is hypothesized that vaccination during homeostasis-driven T cell proliferation, secondary to lymphodepletion, may facilitate education of the T cell REPERTOIRE, resulting in enhanced T cell memory induction and maturation or differentiation of EFFECTOR CELLS to tumor-associated Ags [236, 237]. In clinical studies, the transfer of activated TILs, *ex vivo*-expanded T cells, or vaccination after myeloablative, or non-myeloablative lymphopenia has been shown to induce significant clinical responses in patients with melanoma and non-small cell leukemia [238–241].

Summary

The goal of regulating the host's immune responses, as a THERAPEUTIC STRATEGY for neoplastic, infectious, autoimmune and inflammatory diseases, has been achieved for some indications. Optimism for this approach has fluctuated, but at present, numerous immunoregulatory drugs have been approved, and currently immunotherapeutics represent a quarter of all drug approvals in the United States. During the last decade, we have observed an explosion in the cloning of immunoregulatory genes and their RECEPTORS as well as the development of novel therapeutic approaches. These critical advances represent the culmination of efforts with crude and fractionated NATURAL products, supernatants and cell products.

Over the last 20 years, NONSPECIFIC IMMUNOSTIMULATION has progressed from initial trials with crude microbial mixtures and extracts to more sophisticated uses with a large collection of targeted immune pharmacologically active compounds (only a few of which are discussed here) having diverse actions on the IMMUNE SYSTEM. Further, a body of immunopharmacological knowledge has evolved with these BRMs, which show substantial divergence from conventional pharmacology, particularly in terms of the relationship of dosing schedules to immunopharmacodynamics. This knowledge is important in evaluating agents and predicting appropriate use and EFFICACY. While much remains to be learned and new compounds to be extracted and/or cloned, the future of IMMUNOTHERAPY seems bright. A number of CYTOKINES have been approved, also for numerous supplemental indications [242] in the United States, Europe and Asia. However, it is apparent that combinations of CYTOKINES and BRMs will have optimal activity when used as adjuvants with more traditional therapeutic modalities. Please see the chapter A5 for further details on CYTOKINES.

Selected websites:

The Cytokine Web: *http://cmbi.bjmu.edu.cn/cmbidata/cgf/CGF_Database/cytweb/*

Cytokines Online Pathfinder Encyclopedia: *http://www.copewithCYTOKINEs.de/cope.cgi*

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C7 Anti-infective activity of immunomodulators

K. Noel Masihi

Introduction

Infectious diseases continue to impact human morbidity and mortality. Every individual is vulnerable to microbial infections regardless of socioeconomic status, gender, age group or ethnic background. There has been an explosion of international air travel with an estimated 2 billion passengers travelling on commercial airlines every year. The rapid expansion of globalization and mass tourism has facilitated the spread of disease-causing pathogens from one continent to another at unprecedented rates. This has led to an alarming increase in the number of infectious diseases. According to the World Health Organization, at least 40 new diseases have emerged over the past two decades, at a rate of one or more per year.

An epidemic in one corner of the world may only be hours away from becoming an impending threat elsewhere as was dramatically demonstrated at the beginning of 2009. Viruses such as the pandemic (H1N1) 2009 influenza virus, often labeled as swine influenza virus, and recent H5N1 avian influenza viruses have the capacity to cause global outbreaks in which persons worldwide are at risk for infection and illness. The WHO declared that pandemic (H1N1) 2009 influenza virus had reached pandemic proportions in summer 2009 and in autumn, some Northern hemisphere countries such as the USA declared it as a national emergency. Unprecedented vaccination programs against pandemic (H1N1) 2009 influenza virus were initiated in many EU countries and in North America. Historically, pandemic influenza viruses have caused millions of deaths; the 1918 influenza alone caused over 40 million deaths, more than occurred during the whole First World War. The public health authorities were completely taken by surprise in 2003 when the sudden and unexpected multi-country outbreak of severe acute respiratory syndrome, SARS, occurred. The unfolding tragedy of AIDS, particularly in many developing nations, has been vividly highlighted by the scientific and public media. Tuberculosis, malaria and other infections that caused ravages in the nineteenth century are once again resurgent. Several infectious agents, such as vector-borne West Nile virus in the USA, neurological variants of Creutzfeldt-Jakob disease in Europe, and resistant forms of bacteria in several countries have emerged as public health concerns over the past few years.

Antimicrobial drugs have been instrumental in saving the lives of millions of people worldwide. The effectiveness of many antibiotics is, however, being steadily eroded by the emergence of DRUG-RESISTANT microorganisms [1]. This is evidenced by the adverse effects on the control and treatment of deadly diseases caused by *Mycobacterium tuberculosis* and *Plasmodium falciparum*. Extensively DRUG-RESISTANT (XDR) strains of *Mycobacterium tuberculosis* (MDR-TB and XDR-TB) are raising new challenges in developing countries. The acute respiratory infections in children, mostly caused by *Pneumococci* and *Haemophilus influenzae*, are becoming more drug resistant. Over 90% of *Staphylococcus aureus* strains and about 40% of *Pneumococci* strains are resistant to penicillin, which was introduced in the 1940s. An increasing prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant (MDR) and pandrug-resistant (PDR) *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* is being encountered in the clinics; *Salmonella typhi*, *Streptococcus pneumoniae*, *Enterococcus faecium*, and *Shigella dysenteriae* have been also reported as MDR. Each year, about 25 000 patients die in the EU from an infection with MDR bacteria, and these

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bacteria are causing extra healthcare costs and productivity losses of at least EUR 1.5 billion each year (European Medicines Agency report, 2009, see Selected websites). Crucial drug choices for the treatment of common bacterial, viral, parasitic, and fungal infections are becoming limited or even nonexistent in some cases in the current era of antimicrobial resistance [2]. This development has not been paralleled by an effective increase in the discovery of new medicines for most pathogens, and the rate of new antimicrobials approvals is steadily dropping. The struggle to control infectious diseases, far from being over, has acquired a new poignancy. Novel concepts acting as adjunct to established therapies are urgently needed.

The IMMUNE SYSTEM can be manipulated specifically by vaccination or non-specifically by IMMUNOMODULATION. Immunomodulators include both immunostimulatory and immunosuppressive agents. This chapter concentrates on immunostimulatory agents capable of enhancing host defense mechanisms to provide protection against infections. Synonymous terms for immunomodulators include immunostimulants, immunoaugmentors, BIOLOGICAL RESPONSE MODIFIERS, or immunorestoratives. Their modes of action include augmentation of anti-infectious immunity by the cells of the IMMUNE SYSTEM, encompassing lymphocyte subsets, MACROPHAGES, DENDRITIC CELLS and NATURAL killer (NK) cells. Further mechanisms can involve induction or restoration of immune effector functions and tilting the balance towards cytokine pathways germane to protection. A diverse array of RECOMBINANT, SYNTHETIC, and NATURAL immunomodulatory preparations for prophylaxis and treatment of various infections are available today [3–6]. A concise mind-map of some of the immunomodulators discussed in this chapter is summarized in Figure 1.

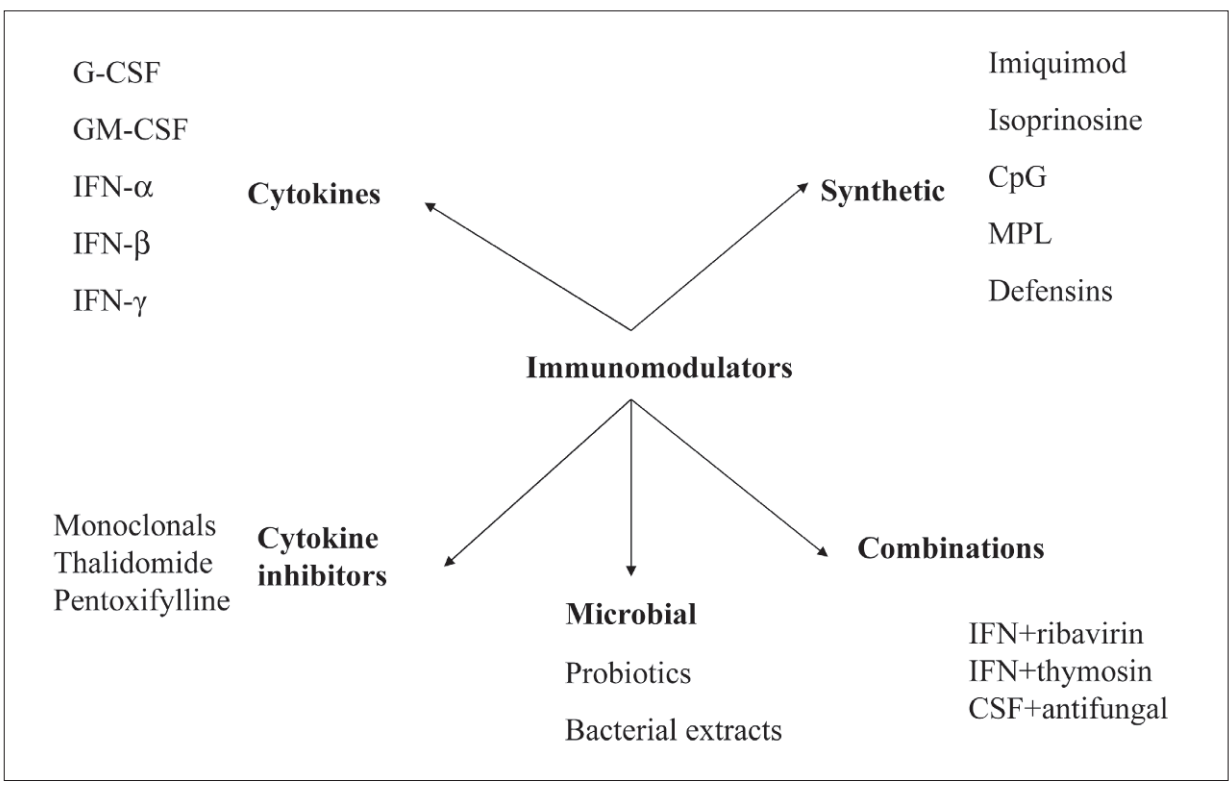

FIGURE 1. BROAD CLASSIFICATION OF IMMUNOMODULATORS

Cytokine immunomodulators

The interactions between a host and an infectious pathogen are complex, diverse and intricately regulated. CYTOKINES, hormone-like polypeptides possessing pleiotropic properties, are crucial in orchestrating the appropriate immune responses critical for the outcome of an infection. Certain CYTOKINES stimulate the production of other CYTOKINES and interact in synergistic or antagonistic networks. CYTOKINES exhibit specific immunomodulatory properties that can enable manipulation of the host response to enhance overall immunogenicity, and direct the nature of the response either toward a type 1 or type 2 pathways. In the type 1 response, Th1 cells produce interferon (IFN)-γ, TUMOR NECROSIS FACTOR (TNF) and INTERLEUKIN (IL)-12 that are required for effective development of cell-mediated immune responses to intracellular microbes. In the type 2 response, Th2 cells produce IL-4, IL-5 and IL-13 that enhance humoral immunity to T-DEPENDENT ANTIGENS and are necessary for immunity to helminth infections.

Recent studies have shown DENDRITIC CELLS (DCs) to be crucial antigen-presenting cells that possess unique T cell-stimulatory capacity. In mouse, lymphoid progenitor-derived DENDRITIC CELLS (DC1) can express IL-12 and preferentially induce type 1 T cell responses, whereas myeloid progenitor-derived DENDRITIC CELLS (DC2) express IL-10 and induce type 2 T cell responses. In humans, DCs are subdivided into plasmacytoid DCs, which secrete copious amounts of IFN-α, and myeloid DCs. Both subtypes of DCs recognize diverse microbial pathogens through specific TOLL-LIKE RECEPTORS (TLR). Local and systemic effects of CYTOKINES are, thus, intimately involved in the host control of infections (Fig. 2) (also see chapters A4, A5, A7). Several RECOMBINANT and NATURAL cytokine

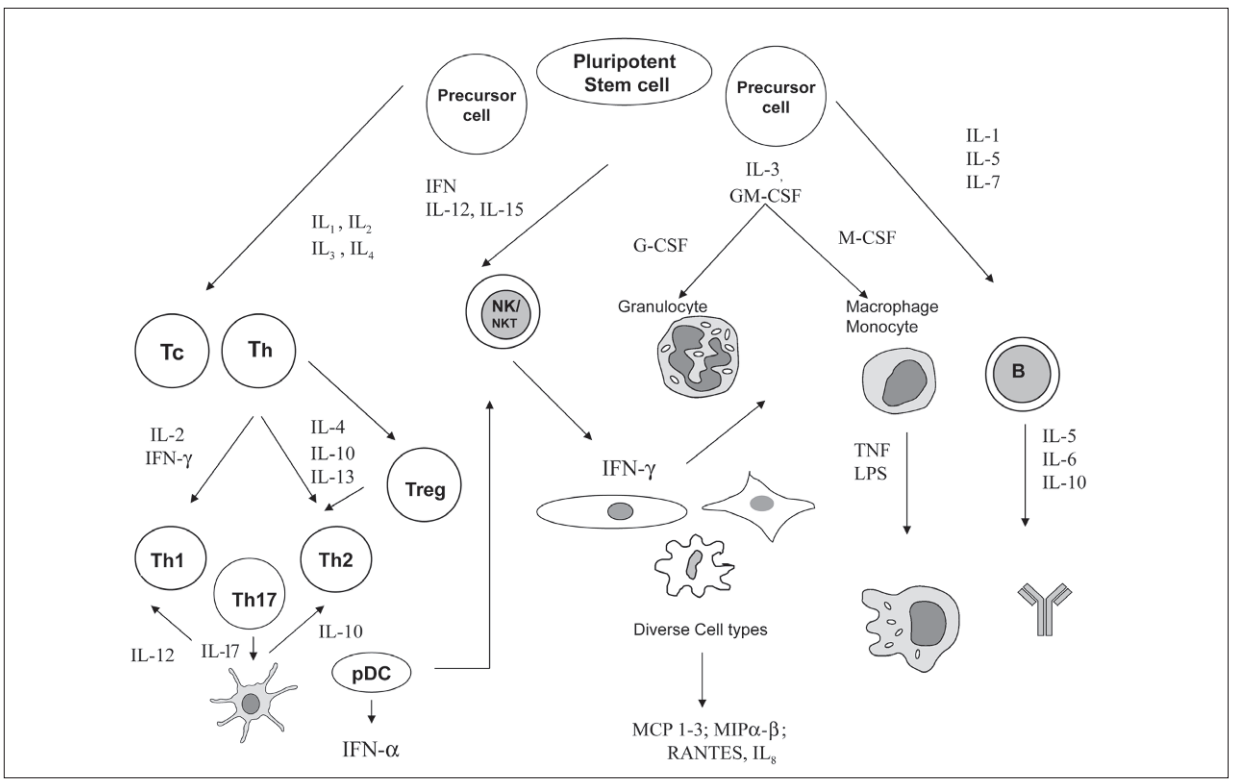

FIGURE 2. INTERACTION OF IMMUNE SYSTEM CELLS AND CYTOKINES

preparations such as, IFNs, and granulocyte colony-stimulating factor (G-CSF) are already licensed for use in patients.

Interferons and combinations

IFNs play an important role in immune activation and mediate an antiviral state that results in impaired viral replication. There are two classes of IFN, type I and type II. The type I IFN is produced in response to a viral infection and includes IFN-α and IFN-β. Most of the IFN-α in human is released by the plasmacytoid DCs, whereas IFN-β is produced by fibroblasts and many other cell types. IFN-α was the first cytokine to be produced by the RECOMBINANT DNA technology. The type II IFN is secreted by activated T cells and NK cells. IFN-γ, known as immune interferon, is a representative of type II IFN. Immunomodulatory IFNs also include omega IFN, tau IFN, asialo IFN and consensus IFN. The mode of action of IFN includes reduction of viral gene protein synthesis, APOPTOSIS, and up-regulation of MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) molecules. The immunity-promoting action of IFN may be a result of direct effect on Th1 differentiation, activation of Stat4 signaling and enhancement of ANTIBODY through stimulation of B and T cells.

IFN-α is a clinically effective therapy used in a wide range of viral infections besides its application in malignant melanoma, basal cell carcinoma and warts. NATURAL IFN-α obtained from human serum and LEUKOCYTES is currently licensed for the treatment of a rare form of cancer, hairy cell leukemia. RECOMBINANT IFN-α2a is licensed for treatment of chronic active hepatitis B and for HEPATITIS C VIRUS infections.

Standard IFN-α has the drawbacks of a short serum half-life and rapid CLEARANCE. To overcome this problem, PEGYLATED forms of IFN have been developed and tested clinically (Box 1). PEGYLATED IFN-α2b (PegIntron) is formed by covalent conjugation of a 12-kDa monomethoxy polyethylene glycol molecule to IFN-α2b, and PEGYLATED IFN-α2a (Pegasys) by covalent conjugation of a 40-kDa branched monomethoxy PEG molecule to IFN-α2a. PEGYLATED IFN-α2b has a prolonged serum half-life (40 hours) relative to standard IFN-α2b (7–9 hours). The greater polymer size of PEGYLATED IFN-α2a acts to reduce glomerular filtration, markedly prolonging its serum half-life (72–96 hours) compared with standard IFN-α2a (6–9 hours) [7].

Hepatitis B

Hepatitis viruses cause INFLAMMATION of the liver. There are five types of hepatitis viruses as is shown in Table 1. Hepatitis A, hepatitis B, and hepatitis C are the most common types. HEPATITIS B VIRUS (HBV) infection is widespread throughout the world, especially in developing nations, and is considered to be a serious global health problem. HBV is HETEROGENEOUS and has eight genotypes, A–H. Genotype D is more prevalent in Mediterranean countries, whereas genotype A is frequent in Western countries. Patients infected with genotype C have more severe outcome of chronic liver disease than those infected with genotype B, particularly in Asia, who seroconvert earlier than those infected with genotype C. Trans-

BOX 1. PEGYLATED INTERFERONS

Pegylated IFN-α2a is formed by covalent conjugation of a 40-kDa branched monomethoxy PEG molecule to IFN-α2a.
Pegylated IFN-α2b is formed by covalent conjugation of a 12-kDa monomethoxy polyethylene glycol molecule to IFN-α2b.
Greater polymer size of pegylated IFN reduces glomerular filtration.
Serum half-life is prolonged from 6–9 hours to 72–96 hours.
Pegylated IFN is injected only once weekly in contrast to multiple administrations necessary for standard IFN, improving patient compliance.

Table 1. Hepatitis viruses

Hepatitis virus	Source of infection	Comments
Hepatitis A virus	Contaminated food	Most common type, vaccine available
Hepatitis B virus, (serum hepatitis)	Blood products, body fluids	Highly contagious, causes liver cancer, vaccine available
Hepatitis C virus	Infected blood, blood transfusion	Chronic liver infection
Hepatitis D virus, (delta hepatitis)	Infectious blood	Incomplete virus, infection only in presence of hepatitis B, common in Mediterranean region
Hepatitis E virus	Fecal matter, contaminated water	Endemic in South America and India

mission is perinatal in areas of high prevalence and sexual or parenteral in regions with low prevalence. The virus, transmitted by blood or body fluids, is up to 100 times more infectious than HUMAN IMMUNODEFICIENCY VIRUS (HIV). HBV infects around 400 million people and kills between 1 and 2.5 million people a year. Overall, 15–25% of HBV carriers die from chronic hepatitis, cirrhosis or hepatocellular carcinoma (HCC). HCC accounts for up to 90% of all liver cancers, and individuals who carry HBV have a greater than 100-fold increased relative risk of developing HCC. The development of HBV to HCC is due to the activation of a signaling pathway that includes the protein β-catenin. Around 50–70% of all HCC tumors show an abnormal accumulation of this oncoprotein within the cell.

At present, the three licensed therapies widely used for treating liver disease caused by HBV are IFN-α (Box 2), lamivudine and adefovir. A further potent inhibitor of HBV DNA polymerase, entecavir, was approved in 2005 by the US FDA. Lamivudine gets incorporated into growing DNA chains and leads to premature chain termination. Adefovir is an oral adenosine monophosphate (nucleotide) analogue that acts by inhibiting reverse transcriptase and DNA polymerase activity of HBV. Entecavir (Baraclude) is an orally administered cyclopentyl guanosine analogue that is rapidly phosphorylated to the active intracellular 5'-triphosphate form capable of inhibiting replication of HBV at three different steps. Most of these therapies are limited in the clinic by a low response rate in terms of loss of hepatitis Be antigen (HBeAg), NORMALIZATION of serum transaminase levels and loss of HBV DNA. Only a small subset of patients with hepatitis B and around 40% of cases with hepatitis C are generally responsive to IFN therapy. IFN-α is also approved for treating conyloma acuminata caused by human papilloma virus and for Kaposi sarcoma in patients with HIV INFECTION.

Long-term treatment with lamivudine or adefovir can result in selection of DRUG-RESISTANT mutants, while troublesome side effects limit the use of IFN-α. Adefovir has been shown to be effective in suppressing lamivudine-resistant HBV. Several promising studies have shown the effectiveness of adefovir and PEGYLATED IFN-α2a and lamivudine-interferon COMBINATION THERAPY and it is gaining increasing favor in the treatment of chronic HBV [8]. PEGYLATED IFN-α2a plus adefovir or lamivudine has been shown to offer a superior protection [9, 10] and to be of a better cost-benefit value. Besides PEGYLATED IFN [11], newer drug formulations demonstrated in clinical trials to be active against HBV include tenofovir, telbivudine and clevudine.

Thymosin-α1 is a SYNTHETIC immunomodulator that mimics thymic-derived thymosin [12] and is approved in over 35 countries for the treatment of viral hepatitis. Thymosin-α1 influences T cell maturation, production of Th1 CYTOKINES, and activity of NK cell-mediated CYTOTOXICITY. Thymosin-α1 stimulates

Box 2. Response to IFN treatment in patients with hepatitis B and C

Hepatitis B virus has eight genotypes, A–H.
Patients with genotype A, B and C give sustained response to PEG-IFN-α when they have high transaminases or low virus levels.
Genotype D patients are more prevalent in the Mediterranean region and have a lower chance of sustained response to PEG-IFN-α.

Hepatitis C virus has six genotypes, 1–6.
Genotype 1and 4 are resistant to IFN therapy, whereas genotypes 2 and 3 are more responsive.
IFN-α2b plus ribavirin combination therapy is effective in chronic hepatitis C virus patients with genotypes 2 and 3.
Treatment duration is 48 weeks for genotype 1 but only 24 weeks for other genotypes.

maturation of CD34 stem cells into $CD3^+CD4^+$ cells and induces increased IL-7 synthesis and GM-CSF, IFN-α, IFN-γ and IL-2 production. Patients with chronic hepatitis B treated with thymosin-α1 had augmented NKT cells and $CD8^+$ cytotoxic T lymphocytes in the liver [13]. Forty-eight weeks after thymosin-α1 treatment some patients showed normalized ALT and decreased HBV-DNA to undetectable level from serum. The lamivudine and thymosin-α1 combination treatment was shown to be superior to lamivudine monotherapy and gave better virological response, HBeAg seroconversion and biochemical response [14]. The combination of thymosin-α1 and IFN-α has been used in patients affected by chronic B and C hepatitis including IFN-non-responders [15]. The combination of thymosin-α1, ribavirin and either pegylated IFN-α, -α2a, -β and -γ are stated to simultaneously and substantially reduce or eliminate the side effects normally associated with the administration of IFN alone. A number of studies have shown that genotype B is associated with a higher response rate to thymosin-α1 therapy than genotype C.

Hepatitis C

Hepatitis C virus (HCV) is the major ecological agent of post-transfusion and community-acquired non-A, non-B hepatitis worldwide. It is estimated that over 200 million people worldwide are infected by the virus. HCV infection is a common cause of chronic viral liver disease and is a leading indication for liver transplantation. Almost all such patients show recurrent hepatitis C viremia. Progressive fibrosis and cirrhosis after liver transplantation have been observed with some of the patients progressing to cirrhosis within 5 years of transplantation. Combination therapy with pegylated IFN and ribavirin (Pegetron) is the current standard of care for the treatment of chronic hepatitis C (CHC) infection. The mechanisms for the observed synergistic effects of combination of pegylated IFN-α and ribavirin have not been elucidated in detail, but in addition to direct antiviral mechanisms, the immunomodulatory effects of both drugs seem to be important, with a shift from Th2 to Th1 cytokine profiles in successfully treated patients. HCV has six genotypes, 1–6, multiple subtypes and quasi-species (Box 2). Genotype 1 is considered to be the most resistant to therapy, whereas genotypes 2 and 3 are more responsive to therapy; genotype 4 seems to be similar to genotype 1 in this respect. IFN-α2b plus ribavirin combination therapy has been found to be effective in chronic HCV patients with genotypes 2 and 3 who are virological non-responders to IFN monotherapy [7, 16]. The treatment duration is 48 weeks for genotype 1 and 24 weeks for other genotypes.

Pegylated IFN-α2a (40 kDa) has superior virological efficacy to IFN-α2a, and elicits histological improvements in chronic hepatitis C genotype 1 patients, with and without sustained virological response, and is effective in those with liver cirrho-

sis. The addition of ribavirin to PEGYLATED IFN-α2a (40 kDa) further enhances the therapeutic benefit for patients with hepatitis C [17]. Once-weekly dosing with either PEGYLATED IFN-α2a or PEGYLATED IFN-α2b has been shown to produce significantly higher rates of viral eradication than standard thrice-weekly IFN-α therapy. With respect to the treatment of CHC, the greatest anti-HCV EFFICACY has been achieved with the combination of once-weekly PEGYLATED IFN and ribavirin [7]. In a clinical study to assess the sustained loss of serum HCV-RNA 12 weeks post-treatment, a regimen employing ribavirin plus PEGYLATED IFN-α2b for 48 weeks gave a successful response in 54% of patients, with a 41% response in those with HCV genotype 1.

IFN-β obtained from human FS-4 fibroblast cell lines is licensed for use in severe uncontrolled virus-mediated diseases in cases of viral encephalitis, herpes zoster and varicella in immunosuppressed patients. A further indication is viral infection of the inner ear with loss of hearing. The standard treatment for MULTIPLE SCLEROSIS, a disease without definitively elucidated etiology, is currently IFN-β.

IFN-γ is the major mediator of host resistance during the acute and chronic phases of infection, and is pivotal in protection against a variety of intracellular pathogens. IFN-γ is primarily produced by T cells, NK cells, and NKT cells. IFN-γ is produced by both $CD4^+$ and $CD8^+$ T cells and can induce MHC class I and class II products. The MECHANISMS OF ACTION that distinguish IFN-γ from type I IFNs are in stimulation of DCs and MACROPHAGES to up-regulate major MHC molecules to enhance antigen presentation and increase expression of COSTIMULATORY MOLECULES. IFN-γ-stimulated MACROPHAGES can produce reactive nitrogen intermediates. NK cells secrete IFN-γ early on during an infection and so facilitate immune cell recruitment and activation, enhancing NK cell CYTOTOXICITY and cell mediated immune responses. Additionally, IFN-γ recruits NEUTROPHILS, and up-regulates CHEMOKINES and ADHESION MOLECULES, and triggers rapid superoxide production and RESPIRATORY BURST. Patients with chronic granulomatous disease are unable to generate an oxidative RESPIRATORY BURST. As a consequence, they develop recurring catalase-positive bacterial infections such as *S. aureus*, *Pseudomonas cepacia*, and *Chromobacterium violaceum*. Multicenter clinical trials have shown that sustained administration of IFN-γ to chronic granulomatous disease patients markedly reduced the relative risk of serious infection. IFN-γ is licensed as a therapeutic adjunct for use in patients with chronic (septic) granulomatosis for reduction of the frequency of serious infections. Imukin and Actimmune™ are IFN-γ preparations marketed for chronic granulomatous disease, mycobacterial and fungal infections.

The major side effects of all interferon therapies include flu-like syndromes, fever, myalgia, headache and fatigue. Hypotension, granulocytopenia, and thrombocytopenia can also occur. Deleterious effects on CENTRAL NERVOUS SYSTEM (CNS), particularly at high doses, have been observed.

Colony-stimulating factors

Granulocyte colony-stimulating factor (G-CSF) preparations such as Filgrastim (r-metHuG-CSF) can significantly enhance neutrophil functions. Filgrastim induces neutrophil production within the BONE MARROW by stimulating the proliferation, differentiation and survival of myeloid progenitor cells. A high incidence of NEUTROPENIA is seen in HIV-infected patients, which considerably increases the risk for bacterial and fungal infections. G-CSF preparations such as Filgrastim can significantly enhance neutrophil functions in patients with AIDS [18] and reverse NEUTROPENIA associated with HIV and cytomegalovirus (CMV) infections. Filgrastim has been granted license extension to cover the treatment of persistent NEUTROPENIA at an advanced stage of HIV INFECTION. In one study, Filgrastim-treated patients have been shown to have 54% fewer severe bacterial infections and 45% fewer days in hospital for any bacterial infections [19]. Determination of absolute numbers of $CD34^+$ progenitor cells and progenitor cell function in HIV-infected patients showed that G-CSF mainly increases the number and differentiation of myeloid progenitors. In another study conducted at 27 European centers on AIDS patients with CMV infection, G-CSF (LENOGRASTIM) was found to be suitable for the treatment of ganciclovir-induced NEUTROPENIA [20]. Recently, pegfilgrastim, a novel RECOMBINANT human

G-CSF, has been pharmaceutically developed by covalent binding of a polyethylene glycol molecule to the N-terminal sequence of FILGRASTIM.

G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF) are used to reverse leukopenia as adjunctive therapy for HIV-associated infections. The GM-CSF preparation Sargramostim helps overcome defects in neutrophil and macrophage function due to its broad range of effects on LYMPHOCYTES, MACROPHAGES, NEUTROPHILS, and DCs through augmentation of cytokine secretion and up-regulation of MHC class II and accessory RECEPTORS involved in the immune response. It enhances the anti-retroviral activity of zidovudine and stavudine in MACROPHAGES and ameliorates the hematological side effects of these agents. Multiple deficiencies are involved in the progression of fungal infections in cancer patients with or without NEUTROPENIA. Although clinical experience is still limited, G-CSF, GM-CSF, and M-CSF show promise as adjuvant therapy for fungal infections [21].

Chemokines and chemokine modulators

Chemokines have been historically regarded as leukocyte chemoattractants capable of regulating cellular trafficking into inflammatory sites. The sobriquet 'chemokine' is abbreviated from chemotactic CYTOKINES. Accumulating evidence suggests that CHEMOKINES have a broad range of functions including macrophage activation, neutrophil DEGRANULATION, DC maturation, T cell activation and B cell ANTIBODY CLASS SWITCHING. Thus, CHEMOKINES can influence both the innate and acquired phases of an immune response. Chemokines can be mediators of angiogenesis and also play important roles in the development of the immune, circulatory and CNS. The major classes of CHEMOKINES comprise the CXC or α CHEMOKINES, the CC or β CHEMOKINES, the C or γ CHEMOKINES, and the CX3C CHEMOKINES. The chemokine family has expanded to 80 ligands including CXCL, CCL, XCL and CX3CL CHEMOKINES and chemokine RECEPTORS including CXCR, CCR, XCR and CX3CR have been described in Table 2.

Many erudite reviews on HIV/AIDS etiology and manifestation have been published and these are not be dealt with here (see the recommended thebody.com website). It is noteworthy that currently there are more women worldwide who have been infected with HIV and account for nearly half of the over 40 million people living with HIV [22]. Women are more vulnerable to HIV INFECTION than men and their increased susceptibility has been linked to the use of hormonal contraceptives and sexually transmitted diseases. Also, susceptibility to HIV varies throughout a women's reproductive life; adolescent girls appear to be most vulnerable to HIV due to high-risk sexual behavior and a not fully mature reproductive system. In addition, recent studies have indicated increases in the risk of acquiring HIV during pregnancy and during the early postpartum period, part of which could be attributed to higher levels of progesterone. Successful control of the HIV pandemic requires continuing focus on gender. Some of the fundamental issues of HIV transmission have been riddled with problems as it is neither feasible to obtain nor examine relevant cells and tissue at the PRECISE time of HIV acquisition.

HIV can infect a wide range of human cells, but has a particular tropism for $CD4^+$ T cells and the monocyte-macrophage cell lineage [23]. HIV induces T cell dysfunction and $CD8^+$ T cell APOPTOSIS, decreasing the number of T cells, which ultimately leads to immunodeficiency. B cell dysfunction can be caused by HIV and is characterized by hypergammaglobulinemia, polyclonal activation, and absence of specific ANTIBODY responses. HIV entry into the host cell is mediated through the CD4 RECEPTOR and a variety of coreceptors. Chemokine RECEPTORS – mainly CCR5 and CXCR4 – have been discovered to be necessary as coreceptors for HIV entry [24].

The binding of the HIV envelope glycoprotein gp120 to CD4 and appropriate chemokine RECEPTOR triggers conformational changes facilitating the fusion of the viral and host cell membranes. HIV found in the vaginal and rectal mucosa is mainly CCR5 dependent [25]. Macrophage-tropic (R5) HIV variants predominantly make use of the CCR5 coreceptors [26]. The T cell-tropic (X4) and dual-tropic (R5X4) HIV strains, generally associated with the clinical manifestations of AIDS, emerge after a latency of several years, although pathogenesis of the

Table 2. Chemokine receptors and ligands

Chemokine receptors	Ligands	Some original names
CC family		
CCR1	CCL3, 3L1, 5, 7, 14, 15, 16, 23	MIP-1α, MCP-3, HCC-2, HCC-4
CCR2	CCL2, 7, 12, 13, 16	MCP-1, MCP-3, MCP-2, MCP-5, MCP-4
CCR 3	CCL5, 7, 8, 11, 13, 24, 26, 28	RANTES, MCP-3, MCP-2, Eotaxin
CCR4	CCL17, 22	TARC, MDC
CCR5	CCL3, 3L1, 4, 5, 8, 14	MIP-1α, MIP-1β, RANTES
CCR6	CCL20	MIP-3α
CCR7	CCL19, 21	MIP-3β, SLC
CCR8	CCL1, 16, 17	TCA3, HCC-4, TARC
CCR9	CCL25	TECK
CCR10	CCL27, 28	CTACK ,CCL28
CXC family		
CXCR1	CXCL6, 8	GRO-α, NAP-2, IL-8,
CXCR2	CXCL1, 2, 3, 5, 6, 7, 8	GRO-α, GRO-β, GRO-γ, IL-8
CXCR3	CXCL9, 10, 11	MIG, IP-10, I-TAC
CXCR4	CXCL12	SDF-1
CXCR5	CXCL13	BLC
CXCR6	CXCL16	CXCL16

BLC, B lymphocyte chemoattractant; CTACK, cutaneous T cell-attracting chemokine; GRO, growth-regulated oncogene; MDC, macrophage-derived chemokine; MCP, monocyte chemotactic protein; MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; NAP, neutrophil activating peptide; RANTES, regulated by activation, normal T cell expressed and secreted; SDF, stromal cell-derived factor; SLC, secondary lymphoid-tissue chemokine; TARC, thymus and activation-regulated chemokine; TCA, thymus-derived chemotactic agent; TECK, thymus-expressed chemokine

CNS and related symptoms are normally associated with M-tropic (R5) HIV strains. X4 HIV INFECTION augments the expression of CHEMOKINES such as MIP-1α (macrophage inflammatory protein) and RANTES (regulated upon activation normal T cell expressed and secreted) [27].

HIV INFECTION can be inhibited by CHEMOKINES and chemokine-related molecules that are ligands for RECEPTORS that function as CORECEPTORS [28]. Chemokine RECEPTORS thus represent important targets for intervention in HIV, and the search for molecules that have a therapeutic potential as inhibitors of these RECEPTORS has been intense [29]. Intervention strategies based on chemokine antagonists that could be useful for the therapy of HIV include RECEPTOR-LIGAND interaction, prevention of the chemokine-glycosaminoglycan interaction, interfering with the signaling pathways that are induced upon RECEPTOR activation, and modification of RECEPTOR pathways [30,31].

Targeting CCR5 and CXCR4

The chemokine RECEPTORS CXCR4 and CCR5 are the main CORECEPTORS used respectively by the T cell-tropic (CXCR4-using, X4) and macrophage-tropic (CCR5-using, R5) HIV for cell entry. Several compounds targeting CXCR4 and CCR5 have been advocated recently. The idea for the drug class came from the observation that presence of mutated CCR5 can confer resistance to HIV INFECTION, even after exposure to numerous high-risk sexual partners. Only around 2% of Caucasians carry such a mutation.

Modified CHEMOKINES, e.g., several low-molecular weight CXCR4 and CCR5 antagonistic compounds, with potent antiviral activity have been described. Maraviroc developed by Pfizer is the first-in-class CCR5 antagonist. It was licensed in October 2007 by the FDA for use in HIV treatment-experienced patients harboring only R5 viruses. It has been shown to prevent the binding of endogenous chemokine MIP-1β to the CCR5 RECEPTOR. The mechanism of action of maraviroc is one of allosteric modification within the transmembrane helices to disrupt the interaction between CCR5 and HIV-1 gp120.

Bicyclam AMD3100 targets CXCR4 and has a potent anti-HIV activity against T-tropic viruses. AMD3100 is being tested in phase III clinical trials as a stem cell recruiting agent in transplantation patients with non-Hodgkin's lymphoma or multiple myeloma. A derivative AMD3451, pyridinylmethyl monocyclam, has been synthesized and reported to show dual CCR5/CXCR4 antagonistic activity against both X4 and R5 HIV strains [32]. In a pharmacokinetics and safety study of AMD3100 in 12 healthy human volunteers, subjects tolerated their dose(s) well without any grade 2 toxicity or dose adjustment. Six subjects experienced mild, transient symptoms, primarily gastrointestinal in nature and not dose related. All subjects experienced a dose-related elevation of the white blood cell count, from 1.5 to 3.1 times the baseline, which returned to the baseline 24 h after dosing [33]. AMD3100 has been shown to interfere with a number of physiological processes that are dependent on the interaction of CXCR4 with SDF-1 [34]. Another chemokine (CCR5) inhibitor, aplaviroc or GSK 873140, has been selected recently by GlaxoSmithKline for phase III trials. Other companies working on CCR5 antagonists include Pfizer Inc and Schering-Plough Corp.

Most existing HIV drugs work inside the body's immune cells, after the virus has infected, and can cause anemia, diarrhea and nerve pain. These new drugs could provide an important treatment option for people with HIV/AIDS, by offering a different mode of action and an improved toxicity profile. Both CXCR4 and CCR5 chemokine RECEPTOR inhibitors may be needed in combination and even in combinations of antiviral drugs that also TARGET other aspects of the HIV replication cycle to obtain optimum antiviral therapeutic effects.

RANTES and MIP ligands

Primary isolates of HIV predominantly use chemokine RECEPTOR CCR5 to enter TARGET cells. The NATURAL ligands of CCR5, the β-CHEMOKINES, RANTES, MIP-1α, and MIP-1β, interfere with HIV binding to CCR5 RECEPTORS and decrease the amount of virions entering cells. Productive HIV INFECTION of primary LYMPHOCYTES requires cellular activation, which increases the intracellular cAMP required for efficient synthesis of proviral DNA during early steps of viral infection. Binding of β-CHEMOKINES to cognate RECEPTORS decreases activation-induced intracellular cAMP levels through the activation of inhibitory G proteins. RANTES and similar CHEMOKINES have been shown to exhibit clear-cut suppressive effects on HIV replication, and other CHEMOKINES such as MIP-1α and monocyte chemotactic protein (MCP) frequently show HIV-inhibitory effects [35]. MIP-1α and RANTES can also reduce T LYMPHOCYTE APOPTOSIS in HIV-infected individuals.

A number of experimental approaches directed towards RANTES have been explored. CCR5 can be down-modulated by RANTES and by ANTIBODIES to CCR5 [25]. It is desirable that CHEMOKINES block HIV INFECTION without triggering CCR3- or CCR5-signaling activity. Two analogues showing increased anti-HIV potency (L-RANTES and C1.C5-RANTES) but capable of antagonistic action against RANTES have been synthesized [29]. The N-terminal region of RANTES contains critical determinants not only for the triggering of RECEPTOR-mediated signaling, but also for

the antiviral function. Recombinant RANTES analogues mutated at the N terminus (C1.C5-RANTES and L-RANTES) and a modified form of RANTES, aminooxypentane (AOP)-RANTES have been synthesized that exhibit an increased binding affinity for CCR5, while showing antiviral activity against different CXCR4-negative HIV [36] and mixed infections with clinical HIV isolates [37]. Promising efficacy as a HIV- microbicidal candidate for topical application has been shown by N-terminally modified chemokine PSC-RANTES against R5-tropic HIV-1 strains. PSC-RANTES is fully protective when applied topically in a macaque model [38], and when encapsulated into biodegradable co-polymer poly(lactic-co-glycolic acid) (PLGA) nanoparticles [39]). Other analogues such as 5P12-RANTES and 6P4-RANTES have been synthesized [40] and shown to protect against rhesus HIV vaginal challenge.

Cytokine inhibitors

Several strategies exist for responding to infection. One mechanism by which the host attempts to restrain the infection is through the up-regulation of cytokines. Some cytokines, such as IL-1, IL-6, IL-8, IL-18, and TNF, counteract the challenge by enhancing the disease in an effort to rid the host of infection. Overproduction of proinflammatory cytokines is believed to underlie the progression of many inflammatory diseases including rheumatoid arthritis, Crohn's disease and endotoxin shock. Many infectious diseases, including HIV, influenza H5N1 and malaria can induce deleterious overproduction of proinflammatory cytokines such as TNF-α and IL-1. An attractive therapeutic approach for potential drug intervention in these conditions is the strategic reduction of proinflammatory cytokines

Intense interest has been generated in developing agents that can block the activity of such cytokines. Inhibition of TNF activity has been singularly successful in the treatment of autoimmune diseases (see chapter C15). Monoclonal antibodies including adalimumab (Humira; Abbott), a fully human monoclonal antibody, etanercept (Enbrel; Amgen/Wyeth), a dimeric construct of soluble p75 TNF receptor and Fc region of human IgG1, infliximab (Remicade; Centocor), a chimeric monoclonal antibody, and Certolizumab pegol, humanized anti-TNF Fab' antibody fragment coupled to polyethylene glycol, are being used in the treatment of rheumatoid arthritis. An IL-1 receptor antagonist, Kineret, has also been licensed. Some of these monoclonal antibody products have shown promise for applications in disease management in patients with HIV/AIDS [41].

Another potent TNF inhibitor, thalidomide, has been used in trials in HIV patients [42, 43]. Thalidomide has a chiral center, and the racemate of (R)- and (S)-thalidomide was introduced as a sedative drug in the late 1950s (Fig. 3). It was withdrawn in 1961due to teratogenicity (creating malformation in embryos, from the Greek for "monster") having caused serious birth defects and neuropathy in more than 10 000 babies.

Several mechanisms of action have been proposed for thalidomide: as angiogenesis inhibitor, down-regulator of integrin, and agent that can reverse the stimulation of insulin-like growth factor I (IGF-I) and fibroblast growth factor 2 (FGF-2) in early limb development. Structural analogues of thalidomide with improved TNF-α inhibitory activity are currently being developed. Pentoxifylline, a methylxanthine usually used in the treatment of peripheral arterial circulatory disorders, has been shown to inhibit TNF synthesis. Currently, clinical trials are ongoing with phosphodiesterase inhibitors and small-molecule inhibitors of TNF-converting enzyme (TACE) that specifically interrupt the signaling pathways of TNF.

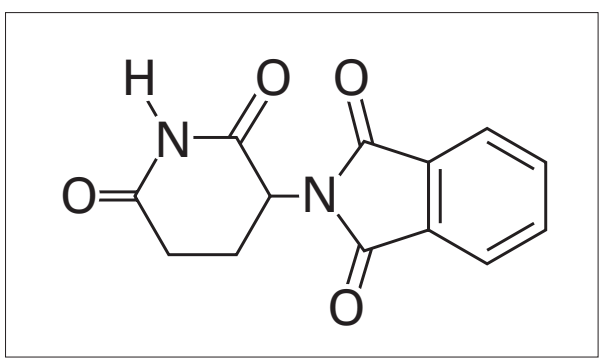

Figure 3. Structure of thalidomide

TNF plays an important role in defense against bacterial and viral infections. Consequently, many patients on anti-TNF drug therapy have shown severe infections such as tuberculosis [44, 45]. A variety of other opportunistic fungal infections by *Candidia*, *Aspergillus Pneumocystis*, *Coccidioides*, and *Histoplasma*, have also been reported in patients treated with anti-TNF monoclonals [46].

Sepsis is one of the most common complications in surgical patients and one of the leading causes of mortality in intensive care units. Sepsis can be caused by infection with Gram-negative bacteria, GRAM-POSITIVE BACTERIA, fungi, or viruses. Sepsis may, however, also occur in the absence of detectable bacterial invasion. In such cases, microbial toxins, particularly Gram-negative bacterial ENDOTOXIN, and endogenous cytokine release have been implicated as initiators and mediators. Septic SHOCK represents the most severe form of host response to infection. Despite recent progress in antibiotics and critical care therapy, sepsis is still associated with a high mortality rate (~40–50%). A number of therapies delaying the onset, and/or reducing the effects of proinflammatory CYTOKINES induced during sepsis are under development [47]. TAK-242 is a small molecule antagonist that reduces LIPOPOLYSACCHARIDE (LPS)-induced production of proinflammatory IL-1, IL-6 and TNF, and is currently undergoing phase III evaluation. Another compound, E5564, or eritoran, is a SYNTHETIC lipodisaccharide and was observed to reduce TNF and IL-6 levels after LPS administration [48] (Fig. 4). Eritoran is being evaluated in a phase III study in patients with onset of severe sepsis. Long-term antagonistic activity can be obtained when eritoran associates with LDL, triglyceride-rich lipoproteins and albumin.

Synthetic immunomodulators

Microbial pathogens possess a variety of evolutionarily conserved structural motifs known as PATHOGEN-ASSOCIATED MOLECULAR PATTERNS (PAMPs). The PAMPS are recognized by a family of specific TOLL-LIKE RECEPTORS (TLRs) that are present on the cells of the IMMUNE SYSTEM such as DCs and MACROPHAGES and play a crucial role in innate immune responses (Tab. 3). Around 13 TLRs exhibiting distinct LIGAND specificities have been identified in humans. TLR2 recognizes bacterial PEPTIDOGLYCAN and lipopeptide, TLR3 recognizes double-stranded RNA, TLR4 binds to LPS, TLR5 binds to flagellin, which is part of the flagellum that propels many kinds of bacteria. TLR7 and TLR8 recognize imidazoquinoline compounds and single-stranded (ss) RNA from viruses, whereas TLR9 binds to unmethylated CpG DNA motifs frequently found in the genome of bacteria and viruses, but not vertebrates. The extracellular portions of microbes are recognized by TLRs, whereas the intracellular

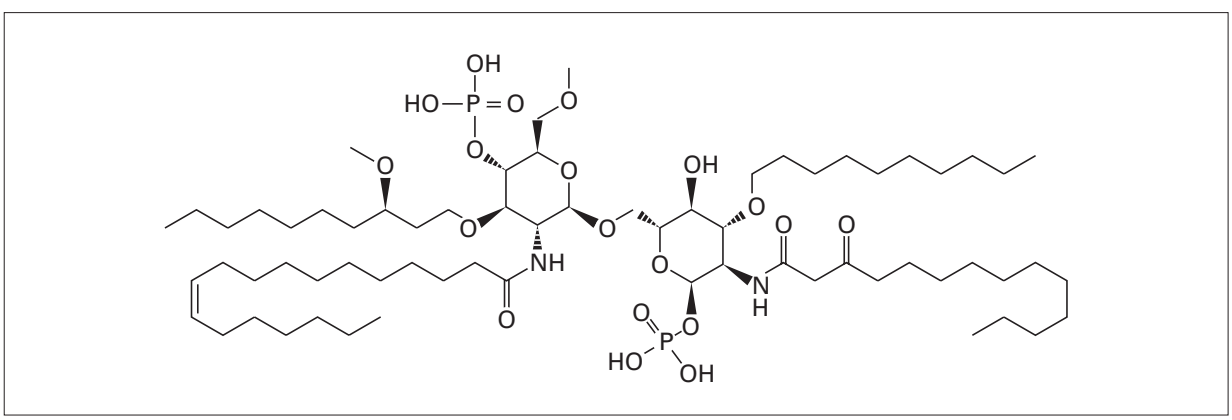

FIGURE 4. STRUCTURE OF ERITORAN

Table 3. Toll-like receptors

TLR	Ligand	Relevant microbial or synthetic source
TLR1	Peptidoglycan, lipopeptide	Gram-positive bacteria
TLR2	Lipoprotein, lipoteichoic acid	Measles virus hemagglutinin protein, RSV
TLR3	dsRNA	CMV, West Nile virus
TLR4	Lipopolysaccharide, lipoteichoic acid, RSV fusion protein	Gram-negative bacteria, RSV
TLR5	Flagellin	Flagellated bacteria
TLR6	Lipoprotein	Bacteria
TLR7	Imiquimod, dsRNA	Synthetic compounds, Influenza virus, HIV
TLR8	Imiquimod, thiazoloquinolones, ssRNA	Synthetic compounds
TLR9	Unmethylated CpG	Bacteria, synthetic ODN
TLR10	?	
TLR11	Profilin	*Toxoplasma gondii*, uropathogenic bacteria
TLR12	?	
TLR13	?	

microbial components are sensed by Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs).

In humans, TLR7 and TLR9 are expressed on the plasmacytoid DC, which can rapidly synthesize large amounts IFN-α and IFN-β in response to viral infection. These observations indicate that TLR3, TLR7 and TLR9 may play an important role in combating viral infections. Interestingly, TLR7 recognizes synthetic immunomodulators such as imidazoquinolone compounds that are used against viral infections.

Imiquimod derivatives

Imiquimod is a fully synthetic immune response enhancing imidazoquinoline amine, (S-26308, R-837) {1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4 amine} [49] and is a TLR7 agonist. Imiquimod is marketed as Aldara™ for genital warts caused by human papillomavirus (HPV) subtypes 6 and 11, but is widely used for basal cell carcinoma, actinic keratosis and molluscum contagiosum. It has shown promise in lentigo maligna and cutaneous metastases of malignant melanoma. Imiquimod and its derivatives such as resimiquimod (R-848) improve antigen presentation by DCs and also act on B cells leading to the synthesis of antibodies such as IgG2a (Fig. 5).

These agents activate macrophages and other cells *via* binding to cell surface receptors, such as TLR7, inducing secretion of proinflammatory cytokines, e.g., IFN-α, TNF-α and IL-12. The presence of this cytokine milieu biases towards a Th1-type immune response and has been exploited clinically in the treatment of viral infections (HPV, herpes simplex virus, molluscum contagiosum). In randomized, double-blind, placebo-controlled studies, imiquimod cream has been shown to be significantly effective in eliminating genital warts in patients with clinical, histopathological and polymerase chain reaction confirmed diagnosis of HPV infection and in treatment of external genital and perianal warts. Topically applied imiquimod cream reduced wart area in HIV-infected patients.

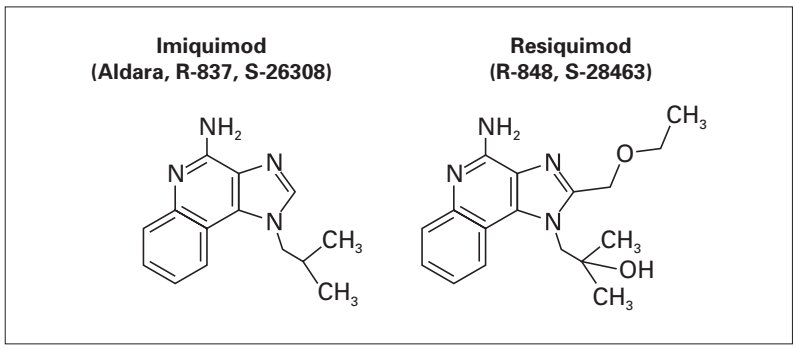

Figure 5. Structures of imiquimod and resiquimod

Isoprinosine

Isoprinosine (Inosiplex) is a complex of the *p*-acetamidobenzoate salt of *N*,*N*-dimethylamino-2-propanol: inosine in a 3:1 molar ratio (Fig. 6).

It is a white crystalline powder soluble in water. The inosine portion of isoprinosine is metabolically labile and half-life in rhesus monkeys has been found to be 3 minutes after intravenous and 50 minutes after oral administration. Isoprinosine has been shown to augment production of cytokines such as IL-1, IL-2 and IFN-γ, inducing a Th1 bias. It increases proliferation of lymphocytes in response to mitogenic or antigenic stimuli, increases active T cell rosettes and induces T cell surface markers on prothymocytes. Isoprinosine is currently licensed in Europe for treatment of herpes simplex infections, subacute sclerosing panencephalitis, acute viral encephalitis caused by herpes simplex, Epstein-Barr and measles viruses and for treatment of these viral infections in immunosuppressed patients. Isoprinosine has been reported to have minor CNS depressant but no neuromuscular, sedative, or antipyretic activities in pharmacological studies in animals. In humans, isoprinosine may cause transient nausea and a rise in uric acid in serum and urine at high doses.

Emerging therapies with synthetic immunomodulators

CpG oligodeoxynucleotides

Over the last decade, nucleotide sequences containing non-methylated cytosine phosphate–guanosine

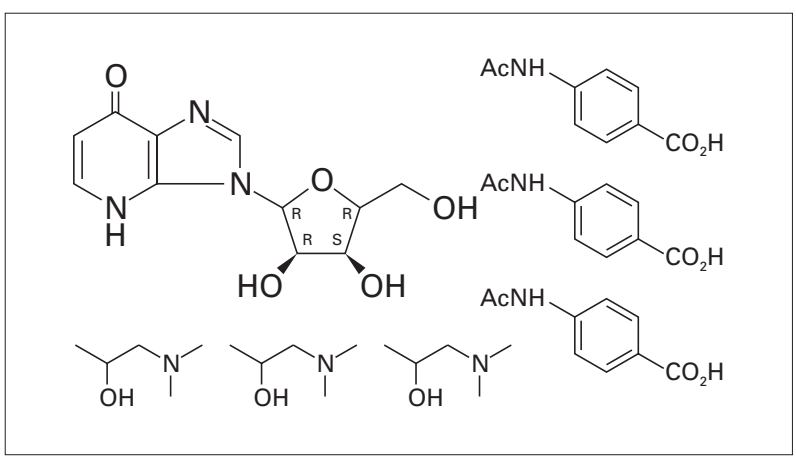

Figure 6. Structure of isoprinosine

(CpG) dinucleotides, with flanking regions of two 5' purines and two 3' pyrimidines, have been discovered to play an important role in INNATE IMMUNITY (Fig. 7).

The CpG motifs in bacterial or viral DNA are detected by TLR9 principally by recognizing differences in the chemical structure between microbial and host DNA. In humans, TLR9 is expressed predominantly in B cells and plasmacytoid DCs.

Although DNA containing CpG motifs (CpG DNA) evolved as a defense system in eukaryotes to probably protect against infection, it is possible to use CpG DNA as an immunomodulator for therapeutic applications. CpG DNA triggers a predominantly Th1 pattern of immune activation and is of interest for the induction of antimicrobial effects. CpG DNA triggers humoral B cell responses and also activates MONOCYTES and MACROPHAGES to secrete CYTOKINES, especially IL-12, TNF, and IFN. Of particular interest is the capability of CpG DNA to overcome the Th2 bias associated with some diseases and in very young and elderly mice [50, 51]. The exact mechanisms by which CpG DNA induces DCs, MACROPHAGES and NK cells to produce immunomodulating CYTOKINES are being elucidated.

The finding that SYNTHETIC oligodeoxynucleotides (ODNs) can mimic the action of bacterial DNA has galvanized research in this area. Also, several viruses such as influenza A virus, HSV, CMV, respiratory syncytial virus (RSV) and vesicular stomatitis virus have been shown to activate cells *via* TLR family members. Administration of SYNTHETIC ODNs containing CpG motifs has been shown to confer protection or act as an adjuvant in experimental infections by influenza virus, hepatitis virus, *Listeria monocytogenes*, *Francisella tularensis*, *Trypanosoma cruzi*, and *Leishmania*, and markedly increases resistance against acute poly-microbial sepsis [52, 53]. Several human clinical trials have shown that CpG-DNA has low toxicity and is well tolerated [52, 54].

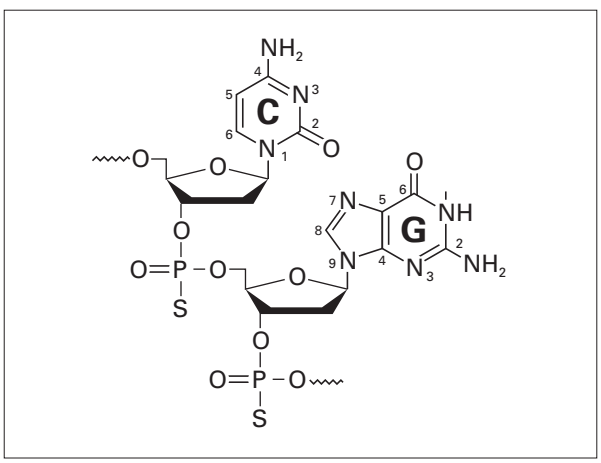

FIGURE 7. GENERAL STRUCTURE OF A CPG-MOTIF

Antimicrobial peptides

ANTIMICROBIAL PEPTIDES (AMPs) also known as host defense peptides (HDPs), are present mainly in phagocytic cells of the IMMUNE SYSTEM and can kill a wide array of Gram-positive and Gram-negative bacteria, enveloped viruses, fungi and parasites through disruption of microbial membranes. The positive charge of AMPs facilitates the interaction with negatively charged surface components, such as LPS in Gram-negative bacterial outer membranes, lipoteichoic acids of GRAM-POSITIVE BACTERIA and heparin sulfate found in viral envelopes [55]. In addition to antimicrobial activity, AMPs also exert immunomodulatory effects through a variety of mechanisms such as CHEMOTAXIS, activation of immature DCs, angiogenesis, and cytokine production [56]. AMPs are genetically encoded and form an important part of the innate immune response (see also chapter A6).

The AMPs are amphipathic cationic molecules of between 10 and 50 amino acids that can be classified into three main groups; α-helical peptides (e.g., cathelicidins), β-sheet peptides (e.g., DEFENSINS); and amino acid-enriched structures (e.g., indolicidin). The structural and functional diversity of AMPs have made them interesting candidates for anti-infective therapy since they have the capability to resolve infections by antibiotic-resistant bacteria [57]. Moreover, they can stimulate innate host defense mechanisms without excessive proinflammatory responses that can be potentially harmful. Two AMPs have been shown to be effective in phase III clinical trials. The α-helical magainin analog, pexiganan, has been developed for TOPICAL treatment of

patients with mild diabetic foot infection and can cure approximately 90% of the patients. The other AMP, omiganan pentahydrochloride (omiganan) is a novel, SYNTHETIC, antimicrobial peptide comprised of 12 amino acid residues with the following primary sequence: ILRWPWWPWRRK-NH_2 (where I is isoleucine, L leucine, K lysine, P proline, R arginine and W tryptophan). Omiganan, is an indolicidin with EFFICACY in preventing catheter-related infections.

SYNTHETIC peptides that retain many of the immunomodulatory properties of naturally occurring HDPs are currently being explored for their therapeutic potential [58, 59]. Innate defense regulator-1 (IDR-1) is an anti-infective SYNTHETIC peptide that selectively modulates the innate immune response. IDR-1 is a 13-amino acid peptide (KSRIVPAIPVSLL-NH_2) synthesized by solid-phase synthesis using standard fluorenylmethoxycarbonyl (FMOC) chemistry protocols. MICROARRAY analysis showed that several transcription factors, including STAT1 and several zinc finger and Hox transcription factors, and ADHESION MOLECULES such as ICAM, NCAM and integrin-α are stimulated by IDR-1. Furthermore, kinase MEK6 (part of the p38 MITOGEN-activated protein kinase pathway) and monocyte-chemotactic-protein (MCP), CHEMOKINES MCP-3 and MCP-1, and CYTOKINES IL-10 and IL-19 are up-regulated. IDR-1 confers protection against multiple bacterial pathogens including strains of methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus* in murine models. IDR-1 has been shown to stimulate monocyte CHEMOKINES, activating monocyte-macrophage cells, and down-regulating proinflammatory cytokine responses without inducing toxic side effects [60].

Defensins

DEFENSINS are a family of structurally related cysteine-rich cationic polypeptides of the INNATE IMMUNE SYSTEM produced in response to microbial infection in humans, animals, insects and plants [61]. Six human α-DEFENSINS have been described; HD1, 2, 3, and 4 are secreted primarily by GRANULOCYTES and certain LYMPHOCYTES, whereas HD5 and 6 are expressed by intestinal Paneth cells. The β-DEFENSINS are produced in response to microbial infection of mucosal tissue and skin (Tab.4). Unusual circular mini-DEFENSINS have also been identified in rhesus monkeys. DEFENSINS of vertebrate animals have been reviewed elsewhere [62]. The mode of action of DEFENSINS includes destabilization and disruption of TARGET cell membranes by strong membrane permeabilizing activities. Permeabilization results in the cessation of RNA, DNA and protein synthesis and with a decreased microbial viability. The human neutrophil DEFENSINS HNP1 and HNP2 exert significant chemotactic effects on MONOCYTES. β-DEFENSINS are chemoattractive for memory T cells and immature DCs and lead to expression of proinflammatory CHEMOKINES and CYTOKINES such as RANTES, macrophage-derived chemokine (MDC), interferon-γ inducible protein 10 (IP-10), MIP-1α and MIP-1β, TNF-α, IL-1 and IL-12.

Recent reports have highlighted the anti-HIV activities of DEFENSINS, whose structure and charge resemble portions of the HIV-1 transmembrane envelope glycoprotein gp41. CD8 T LYMPHOCYTES from certain immunologically stable HIV-infected individuals secrete soluble factors that suppress HIV replication. CD8 T cells from long-term non-progressors with HIV INFECTION were found to secrete a cluster of proteins identified as α-defensin 1, 2, and 3 on the basis of specific ANTIBODY recognition and amino acid sequencing. Interestingly, α-DEFENSINS were shown to effectively suppress HIV replication *in vitro* about 10 years ago and can specifically block the initial phase of the HIV infectious cycle by binding specifically to CD4, and gp120 [63]. A study of seronegative women who were exposed constantly to HIV-1 demonstrated that their $CD8^+$ cells exhibit extensive α-defensin production at both peripheral and mucosal levels. The α-defensin expression level in these seronegative women was tenfold higher than that of control subjects [64]. Likewise, overexpression of α-DEFENSINS in breast milk results in a low rate of HIV-1 transmission from mother to infant [65].

HIV induces β-defensin-2 and -3 in human oral epithelial cells, which exhibit strong anti-HIV activity [66] due to the direct antiviral effect or competition for the chemokine RECEPTORS that HIV uses to enter the cell [67]. Mother-to-child transmission of HIV is the main source of pediatric AIDS. There is a significant relationship between genetic variants of β-defensin-1 gene, viral load, and mother-to-child

Table 4. Human defensins

Defensins	Cell types	Characteristics
α-Defensins		
HNP1–HNP4	Neutrophils	Broad spectrum of antimicrobial activity, chemoattractant for monocytes, T cells and dendritic cells
HD5–6	Intestinal tract	Propeptide released by Paneth cells. Converted to the active form by trypsin
β-Defensins		
hBD-1	Keratinocytes, respiratory epithelia, urogenital tract	Weak antimicrobial activity
hBD-2	Keratinocytes, respiratory epithelia, intestinal tract	Active against Gram-negative bacteria and fungi, chemoattractant for dendritic and T cells, human neutrophils and mast cells
hBD-3	Keratinocytes, respiratory epithelia	Broad spectrum of antimicrobial activity
hBD-4	Keratinocytes, respiratory epithelia	Activity against *P. aeruginosa*, *E. coli* and Gram-positive bacteria

transmission of HIV. In mothers, the -52GG genotype is associated with low levels of HIV plasma viremia and a lower risk of maternal HIV transmission [68].

An antimicrobial peptide homologous to rhesus monkey circular mini-defensins (δ-defensins) has been prepared by solid-phase peptide synthesis and named retrocyclin. Retrocyclin has a remarkable ability to inhibit proviral DNA formation and to protect CD4 lymphocytes from *in vitro* infection by both T-tropic and M-tropic strains of HIV. Retrocyclin interferes with an early stage of HIV infection and retrocyclin-like agents might be useful topical agents to prevent sexually acquired HIV infections. It is of interest that recent studies have shown that human cells possess the capability to make cyclic θ-defensins [69].

Defensins are known to exhibit inhibitory activity against several viruses. α-defensins promote uptake of influenza virus by neutrophils, and human defensins 5 and 6 are effective in neutralizing influenza virus [70]. The expression of murine β-defensin was enhanced in influenza-infected lungs, trachea and nasal mucosa [71] and treatment of cell cultures with human neutrophil peptides soon after infection resulted in marked inhibition of influenza virus replication and viral protein synthesis [72]. BK virus is a polyomavirus that establishes a lifelong persistence in most humans. Studies have shown that human α-defensins can inhibit BK virus infection [73], and inhibit adenovirus infection [74], and that α-defensins and human β-defensin inhibited herpes simplex virus infection [75].

Microbial-derived immunomodulators

Cell walls of Gram-negative bacteria contain endotoxic LPS, which is a potent stimulator of the immune system, even in nanogram quantities. Clinical application of endotoxin has, however, been hampered as it plays a major role in the pathophysiology of gram-negative bacterial sepsis. Concerted efforts have been made to modify endotoxin for possible therapeutic use in humans. Investigations on endotoxic LPS have led to the identification of lipid A as an important constitu-

ent of the ENDOTOXIN molecule capable of exhibiting various immunopharmacological activities of the intact bacterial LPS. During the last few decades, selective reduction of the toxicity of ENDOTOXIN, while retaining its beneficial adjuvant property, has been achieved by effective chemical treatment and by synthesis of non-toxic lipid A analogues.

Monophosphoryl lipid A

Many advances in our understanding the TLR RECEPTORS and associated agonists have been made in recent years. This has led to the engineering of a new generation of adjuvants that incorporate these agonists for prophylactic and therapeutic vaccines. LPS (ENDOTOXIN) is a specific agonist of TLR4 but is too toxic for use in humans. Monophosphoryl lipid A (MPL) is a detoxified derivative of LPS isolated from the Gram-negative bacterium *Salmonella minnesota* and has been shown to be also a TLR4 agonist. MPL promotes IFN-γ production and skews the immune response toward a Th1 profile [76] (Fig. 8).

Formalin-inactivated RSV vaccine induces an immunopathological response that leads to disease enhancement upon RSV infection of those previously vaccinated. Vaccine containing MPL dramatically reduced the levels of Th1 and Th2 CYTOKINES in response to RSV challenge [77]. Prophylactic administration of MPL has been shown to mitigate the sepsis syndrome by reducing chemokine production of pulmonary and hepatic MIP-1α, MIP-1β, MIP-2, and MCP-1 mRNA and attenuating the production of proinflammatory CYTOKINES. MPL has been used as an adjuvant with toxoplasmal, leishmanial, malarial, and tuberculosis ANTIGENS in preclinical studies.

Aluminum salts have long been used as adjuvants. They promote ANTIBODY production and are more biased towards a Th2 response. The particulate structure of aluminum salts facilitates formation of

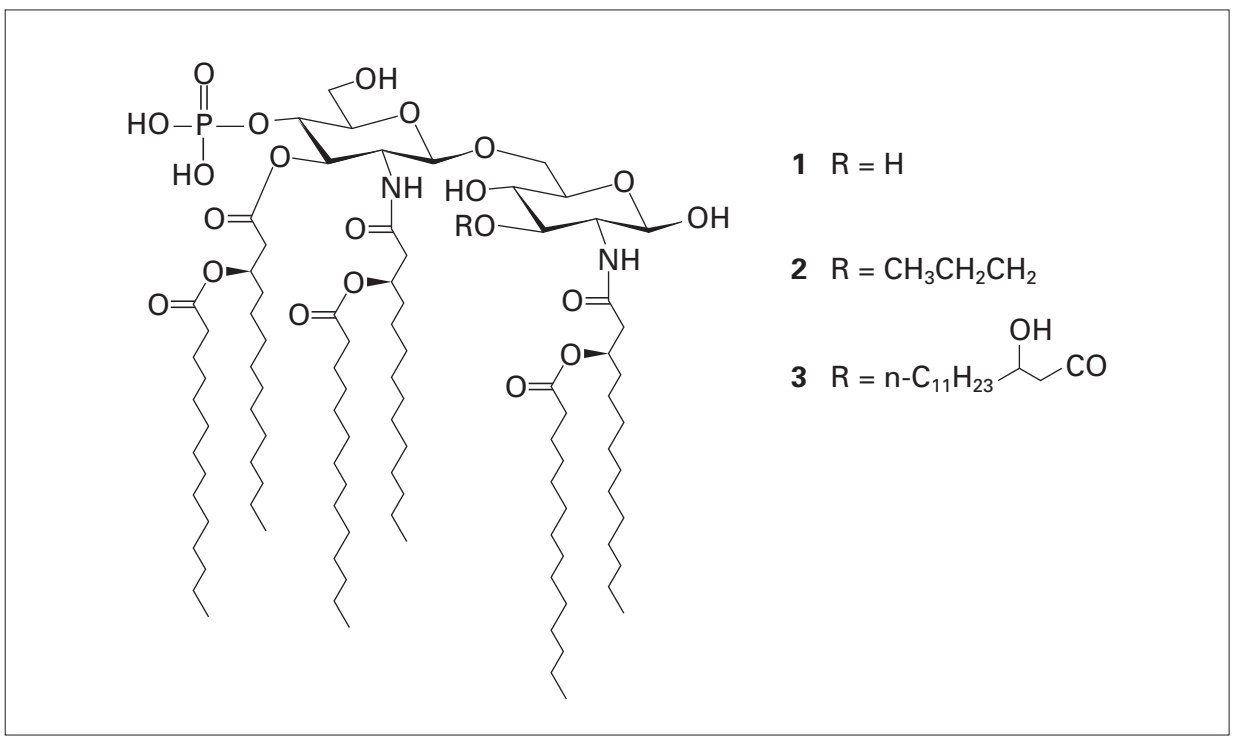

FIGURE 8. STRUCTURE OF MONOPHOSPHORYL LIPID A

an antigen depot at the injection site for a sustained response. At the molecular level, aluminum adjuvants have been shown to stimulate Nlrp3, a component of the INFLAMMASOME. The multifunctional INFLAMMASOME can lead to processing of proforms of cytokine IL-18 or IL-1β. Alum alone is not able to induce the transcription of IL-18 or IL-1β genes in antigen-presenting cells (APCs) and needs additional pretreatment of the APC with TLR ligands such as MPL.

The Adjuvant System 04 (AS04), licensed for use in humans, consists of MPL (3-*O*-desacyl-4'-monophosphoryl lipid A) adsorbed onto a particulate form of aluminum salt. It has been developed by GlaxoSmithKline BIOLOGICALS as a new generation of adjuvants. MPL and aluminum salt apparently synergize in AS04 to produce elevated levels of IL-18 or IL-1β. AS04 is currently used in two licensed vaccines, Fendrix against HBV and Cervarix against HPV-16 and HPV-18 cervical cancer. A third vaccine against herpes simplex 2 virus is in phase III clinical trials.

Microbial immunomodulators

Probiotics

PROBIOTICS are living microorganisms that are ingested by the host on purpose for improving intestinal microbial balance and promoting health. The global interest in innovative PROBIOTICS as a NATURAL way of restoring the body's normal MICROBIOTA, as an alternative way of preventing or treating infectious diseases without side effects and for general well-being, has increased dramatically since the end of the 1990s and the trend remains unbroken. Several probiotic products are available as freeze-dried, lyophilized or heat-dried culture supernatant capsules, and as fortifiers of dairy foods (e.g., yogurt, milk, kefir, ice cream or cheese).

Several immune functions, such as PHAGOCYTOSIS, NK cell activity and mucosal Ig A production (especially in children), can be improved by some probiotic bacteria (see also chapter C8). The major *Lactobacillus* species present in the human gastrointestinal mucosa, *L. plantarum*, *L. rhamnosus* and *L. paracasei*, have been found to be potent stimulators of IL-12 [78]. PROBIOTICS, including lactobacilli and bifidobacteria, administered to children can reduce incidence and duration of diarrhea. *Bifidobacterium breve* has been shown to augment ANTIBODY production and induce significant reduction in the frequency of rotavirus shedding in stool samples of infants [79]. Probiotic supplementation can reduce the risk of travelers' diarrhea in adults [80]. Whole microbes such as *Lactobacillus casei*, *Bifidobacterium* species and *Saccharomyces boulardii* have been used successfully to prevent antibiotic-associated diarrhea, and to treat other diarrheal illnesses caused by bacteria [81]. The beneficial bacterial flora present in the human body can be destroyed by antibiotic treatment and permits overgrowth of pathogenic bacteria. Antibiotics are designed to attack specific bacterial pathogens but, in the process, indiscreetly reduce the number of beneficial human MICROBIOTA that is part of the gut-associated lymphatic system [82]. Thus, supporting normal flora with live microorganisms can confer a beneficial health effect [83].

Probiotic lactobacilli can improve urogenital health by immune modulation, pathogen displacement and creation of conditions that are less conducive to proliferation of pathogens [84]. PROBIOTICS have been used for treating candidal vaginitis and urinary tract infections. Bacterial vaginosis is a common condition that recurs frequently in premenopausal women. It adversely affects women's lives, and is associated with several complications including increased risk of sexually transmitted infections and HIV, and even adverse pregnancy outcome. It is characterized by depletion of the indigenous lactobacilli. The use of PROBIOTICS to populate the vagina and prevent or treat infection has shown EFFICACY, including supplementation of antimicrobial treatment to improve cure rates and prevent recurrences [85]. Probiotic lactobacilli can provide benefits to women being treated with antibiotics [85, 86]. Urinary tract infection in children is common (5–10%) and recurs in 10–30%. PROBIOTICS have also been used in children [87] and have been shown to reduce febrile illness caused by the urinary tract infection [88].

PROBIOTICS may have a beneficial effect on the severity and duration of symptoms of respiratory infections but do not appear to reduce their incidence [89], and probiotic supplementation may

prevent the spread of infection throughout the respiratory tract [90]. Risk of early acute otitis media and antibiotic use and the risk of recurrent respiratory infections during the first year of life may be reduced by PROBIOTICS [91].

PREBIOTICS are non-digestible food ingredients that confer benefits upon host well-being and health, whereas synergistic combinations of pro- and PREBIOTICS are called synbiotics. Treatment with synbiotics has been reported to significantly decrease the risk for sepsis by bloodstream infections [92]. It is interesting that the intestinal MICROBIOTA differs in infants who later develop allergic diseases, and feeding PROBIOTICS to infants at risk has been shown to reduce their rate of developing eczema [93]. Lactobacilli and bifidobacteria have been reported to reduce risk and severity of allergic disease, particular atopic dermatitis [80].

Bacterial extracts

BACTERIAL EXTRACTS have been widely used as immunomodulators to prevent recurrent infections of the upper and lower respiratory tract, particularly in children. They are comprised of common bacterial respiratory pathogens found in the respiratory tract. Several preparations containing bacterial lysates (e.g., Broncho-Munal®, Broncho-Vaxom®, Imocur®, Luivac®) are licensed for use in Europe. Such preparations are, however, regarded with scepticism by many authorities who question their medicinal value.

A large body of primary literature exists on placebo-controlled and double-blind clinical trials that have been conducted with these agents. Patients, most often children, having recurrent episodes of infections of the respiratory tract and of the ear, nose and throat have been treated with oral bacterial lysates. In most cases, the frequency and the severity of infections were reported to be reduced and both the physician and the patient considered the treatment to be beneficial. It is of interest that many studies have reported a decrease in antibiotic consumption in bacterial lysate-treated patients and a reduction in the number and duration of hospitalizations [94, 95]. A multicenter study with the oral bacterial lysate immunomodulator LW 50020, involving 4965 recurrent respiratory tract infection patients in 14 countries in Europe, Latin America, and Asia, has been conducted. An overall reduction of at least 50% in the number, severity, and duration of respiratory tract infections, the number of antibiotic and symptomatic treatments, and the number of days absent from school or work was reported [96]. Hemodialysis patients suffer from several immune defects, which increase their risk of developing bacterial infections, particularly of the respiratory tract. In a double-blind placebo-controlled prospective study, oral IMMUNOTHERAPY with an immunomodulating bacterial extract significantly reduced the number of patients with respiratory tract infections and, consequently, the number of antibiotic treatment courses [97].

Mucosal immunization strategies able to induce secretory IgA for protection of mucosal surfaces and systemic immunity to pathogens invading the mucosal surface of the host are currently of great interest in the field of infectious diseases. The current concept of the mucosal IMMUNE SYSTEM postulates that stimulation of the gut-associated lymphoid tissue (GALT) can lead to the induction of a generalized response by the whole mucosal-associated lymphoid tissue. LYMPHOCYTES that have been sensitized to the antigen in the GALT migrate *via* the blood to mucosal tissues to generate antigen-specific secretory IgA ANTIBODIES, which play a key role in protection against pathogens invading mucosal surfaces [98]. Bacterial lysates such as Broncho-Vaxom® have been shown to increase IgA concentrations [99]. Earlier studies conducted in 1990s and early 2000s already showed that such preparations can enhance the production of diverse CYTOKINES such as IL-1, IL-12, IFN-γ, stimulate NK cells, induce B lymphocyte proliferation and activate phagocytic activity of MACROPHAGES [99, 100]. The main problem with the use of BACTERIAL EXTRACTS may be cutaneous eruptions, itching and increased risk of lower urinary tract infections.

Summary and perspective

Preventing existing and emerging infectious diseases is a multidisciplinary and multifaceted endeavor. The

traditional treatment of infections has focused on antimicrobial agents and on the induction of specific immune defenses. The appeal of immunomodulators is inherent in modes of action that are distinctly different from the direct microbicidal action of established antibiotic, antiviral, antifungal and antiparasitic drugs. The past few years have witnessed an explosion of scientific and clinical advances in the field of immunomodulator biology and therapy. There has been considerable interest in the therapeutic use of biologically manufactured CYTOKINES and cytokine inhibitors in infectious diseases, chronic inflammatory conditions and cancer. The immune-based therapies in combination with antimicrobial drugs, such as IFNs and ribavirin for viral hepatitis, especially, have attracted attention. A significant level of knowledge of the structure-activity relationships of CHEMOKINES has been amassed. Recent research has elucidated the mechanisms underlying the inhibitory activity of CHEMOKINES and has been instrumental in the rational design of anti-HIV chemokine analogues. Activation of innate non-antigen-specific host defenses by recently recognized immunomodulatory agents such as CpG DNA will increasingly find a beneficial role in the prevention of infections. TLR agonists such as MPL are being increasing used. The ongoing and upcoming trials of immunomodulatory agents should provide a wealth of information on clinical and safety matters. It is now also possible to control the deleterious overproduction of INFLAMMATORY CYTOKINES observed in certain disease states with several immunomodulatory agents. The potential market involving CYTOKINES is enormous and is poised to grow steadily. As an example, industry analysts estimate that successful CCR5 drugs for HIV and other disease states could generate sales of $500–700 million a year. It is noteworthy that over 60 biotechnology protein drugs, including the RECOMBINANT proteins and MONOCLONAL ANTIBODIES that are currently available, represent over a quarter of the drugs recently introduced worldwide.

Discovery of new and continued development of diverse immunomodulators could complement established therapies. A greater understanding of the underlying molecular MECHANISMS OF ACTION should enable their future development. The goal still remains to convert the manifold advances made in the immunostimulants field into clinically applicable therapies. A diverse array of structures is available and it seems reasonable to expect that data from relevant infectious disease models will eventually lead to exciting breakthroughs. New methodologies have the potential to identify novel targets and foster the development of individually tailored immunomodulatory drug treatments.

Selected readings

Fallon P. *Pathogen-Derived Immunomodulatory Molecules*. Austin: Landes Biosciences co-published with Springer, Heidelberg, 2009: 1–202

Recommended websites

http://www.who.int/csr/disease/en/
http://www.emea.europa.eu/pdfs/human/antimicrobial_resistance/EMEA-576176–2009.pdf
http://www.thebody.com/index.html
http://www.copewithcytokines.de/cope.cgi
http://www.sabiosciences.com/newsletter/PATHWAYS07_TLR.pdf

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Immunopharmacology of probiotics and prebiotics

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Introduction

The medicinal properties of various nutritional components have been appreciated since ancient times. Hippocrates (460–377 B.C.), for example, stated: "Let medicine be thy food and food be thy medicine". Tea brewed from various fruits, shrubs and trees containing NATURAL salicylates, has been consumed for pain relief since the Stone Age. Also the origin of the most well-known painkiller found in almost every home – aspirin – is a tree extract (see chapter C14). These examples illustrate how nature can provide the chemical structure for a pharmaceutical, and like aspirin, it is now appreciated that almost 70% of current drugs have their origin in chemical compounds found in plants, fruits and vegetables. Modern medicinal chemists are capable of isolating and identifying these active chemical compounds and then modifying them to yield compounds with increased activity and fewer side effects.

There is a large body of evidence indicating that human health is also modulated through interactions with microbes present in the intestine. The beneficial effect of certain microbes was postulated more than 100 years ago by the Nobel Prize winner Elie Metchnikoff, from the Pasteur Institute in Paris, when he stated that certain lactic acid bacteria present in yoghurt were beneficial to health and had life-prolonging properties. This notion has been validated by a broad array of products that aim to improve human health through direct administration of PROBIOTICS or PREBIOTICS. PREBIOTICS are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of a single or limited number of bacterial strains in the colon that can improve host health [1]. PROBIOTICS, according to the FAO/WHO, are "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" [2].

The intestinal microbiota

The intestine is inhabited by a vast number of microbes that form an intimate partnership with the human host, contributing to and influencing many aspects of human health [1]. Gastrointestinal (GI) MICROBIOTA refers to the community of bacteria and other microorganisms in the GI tract, also called resident flora.

This clearly is true for the functioning of the IMMUNE SYSTEM, as the GI tract is the largest immune organ in the body. It contains approximately 65% of the overall immunological tissues and up to 80% of the immunoglobulin (Ig)-producing tissues of the body. These immunological areas in the GI tract are involved in suppressing or moderating the potent anti-microbial arsenal that has evolved to manage explicit pathogenic threats, as well as preventing the induction of overt INFLAMMATION by damage-associated molecular patterns. The colonization and establishment of the gut MICROBIOTA during the neonatal period is crucial for maturation of the intestinal IMMUNE SYSTEM [3], development of intestinal morphology and maintenance of an immunologically balanced inflammatory response. The composition and development of the intestinal MICROBIOTA in babies is influenced by genetic factors, exposure to microbes, and mode of delivery, but also to a large extent by the composition of human breast milk (see below), which in turn contributes to the maturation of the baby's IMMUNE SYSTEM.

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The development of gut microbiota during infancy

Clinical data show that breast-fed children have high numbers of the health-promoting *Lactobacilli* and *Bifidobacteria*, whereas children who are bottle-fed with a cow's milk formula without additional oligosaccharides have significantly lower numbers of these intestinal bacteria [4]. This large impact of breast milk on the infant's MICROBIOTA is important to achieve an immunological balance that is disturbed during pregnancy [5]. To prevent rejection of the fetus, a Th2-skewed environment is created. This phenomenon, together with a highly protective germ-free environment, is probably the reason why babies are born with an immature IMMUNE SYSTEM. This immaturity results in deficiencies of both the innate and adaptive immune responses in early life.

From the moment solid foods are introduced and the dietary intake starts to resemble that of adults, both the MICROBIOTA and the intestinal IMMUNE SYSTEM form important parts of our line of defense against ingested (pathogenic) micro-organisms. In the gut, there is continuous cross-talk between microbes and the intestinal IMMUNE SYSTEM and this requires a delicate integration of pro- and anti-inflammatory signals to regulate innate and adaptive immune responses and control INFLAMMATION.

Microbiota dysbalance and disease

In recent years, it has become clear that many gut-related disorders are correlated with a dysbalance in the MICROBIOTA and undesired activity of the IMMUNE SYSTEM. Well-known examples include inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis [6,7]. In addition, impaired immune functioning is frequently associated with irritable bowel syndrome, which is characterized by the frequent occurrence of gut discomfort symptoms such as diarrhea, constipation or bloating [8–10]. It is also recognized that the MICROBIOTA and general gut integrity play a role in other disease pathologies like chronic heart failure [11], autism [12], ALLERGY [13–15] and HIV [16].

Highly dysbalanced *in vivo* disease models are associated with severe clinical symptoms and often provide good working models. One of the best studied disease models in this field is represented by IBD. There is overwhelming evidence that, due to genetic factors in the host, the intestinal MICROBIOTA drives a chronic and often severe INFLAMMATION. Specific dysbalances occur in the MICROBIOTA and by comparing the microbiotic composition in Crohn's disease patients, it was established that a reduction of a usually abundant member of the *Firmicutes*, *Faecalibacterium prausnitzii*, is associated with a higher risk of postoperative recurrence of Crohn's disease in the ileum [17]. Besides differences in the MICROBIOTA, cytokine and chemokine levels were shown to be strongly elevated in inflamed intestinal regions of IBD patients [18]. In a series of elegant studies *in vitro*, using Caco-2 cells and peripheral blood mononuclear cells (PBMCs), it was shown that *F. prausnitzii* strongly reduces inflammatory responses. Administration in a murine TNBS-induced colitis model resulted in a strong reduction of INFLAMMATION. The overall evidence shows that *F. prausnitzii* is able to reduce INFLAMMATION by blocking NF-κB activation and IL-8 production. These results suggest that counterbalancing this hyper-immune reaction using *F. prausnitzii* or microbes with similar anti-inflammatory activities as a probiotic may be a promising strategy in Crohn's disease treatment.

As mentioned above, the involvement of the MICROBIOTA and general gut integrity also plays a role in disease pathologies, e.g., HIV INFECTION, that are not considered as general gut diseases *per se* [16]. HIV INFECTION is a progressive disease, characterized by a gradual loss of $CD4^+$ T cells and increased immune activation (see chapter A8). The GI tract plays a major role in HIV-induced chronic immune hyperactivation [19]. HIV INFECTION leads to the rapid loss of gut-associated $CD4^+$ T cells, impaired function of epithelial cells and alterations in the composition of the gut MICROBIOTA, leading to a loss of homeostasis in the GI tract and barrier function [20]. As a consequence, bacteria or bacterial products such as lipopolysaccharides (LPS) and peptidoglycans can translocate and enter the circulation, leading

to increased INFLAMMATION and immune activation. Additionally, in the gut-associated lymphoid tissue (GALT), the rapid decrease in $CD4^+$ T cells early in infection is only partially reversible in most patients, even after years of highly active anti-retroviral therapy (HAART) [21, 22]. This suggests limited access of anti-retroviral drugs to the intestinal mucosa, which may serve as a viral reservoir with low levels of sustained, ongoing viral replication. This offers the possibility of reaching and modulating the GALT with targeted nutritional therapies.

Modulation of the immune system

Probiotics and the immune system

The notion that gut microbes actively modulate the IMMUNE SYSTEM has reinforced interest in PROBIOTICS to bring health benefits. Two major MECHANISMS OF ACTION can be distinguished for immune modulation by PROBIOTICS: direct interaction with the intestinal IMMUNE SYSTEM or modification of the gut MICROBIOTA composition and activity. A number of benefits may be achieved through modulation of the IMMUNE SYSTEM, including relief of diarrhea, increased resistance to infections [23, 24], reduction of INFLAMMATION and allergies [25, 26] and an improved response to vaccination [27].

In recent years, there has been huge progress in the field of microbial interactions with the IMMUNE SYSTEM. Several molecular pathways have been elucidated, as exemplified by the identification of bacterial ANTIGENS and their cognate RECEPTORS, such as TOLL-LIKE RECEPTORS (TLRs), as well as signaling pathways in immune cells. The degree of severity of some MICROBIOTA dysbalance diseases has indeed been linked to TLR1, -2 and -6 polymorphisms in ulcerative colitis [28] and to up-regulation of TLR2 and -4 in Crohn's disease [29]. The gut epithelium dampens TLR signaling and/or limits/sequesters TLR expression, but how specific presentation of TLR ligands from probiotic bacteria to mucosal immune cells may enhance local and systemic immune responses remains unclear. However, tantalizing evidence from cell culture models and animal studies shows that ligands from probiotic bacteria may engage specific TLRs to decrease mucosal inflammatory tone.

ADAPTIVE IMMUNITY is also crucial in MICROBIOTA TOLERANCE. Members of the commensal MICROBIOTA are continuously sampled by the M-cells that lie over the PEYER'S PATCHES for processing by local DENDRITIC CELLS and subsequent education of regulatory $CD4^+$ T cell populations (Fig. 1). TOLERANCE results from induction of regulatory T cells that prevent immune responses toward the tolerizing antigen through secretion of inhibitory CYTOKINES [e.g. INTERLEUKIN (IL)-10 and transforming growth factor β (TGF-β)]. This allows the systemic adaptive IMMUNE SYSTEM to remain ignorant of the ongoing interactions with the normal MICROBIOTA and prevents autoimmune responses. Selected PROBIOTICS exhibit anti-inflammatory capacities both *in vitro* and *in vivo* and are able to prime DENDRITIC CELLS to confer a protective anti-inflammatory effect [30]. Another potent mechanism by which the mucosal adaptive IMMUNE SYSTEM can mediate inflammatory and immune TOLERANCE toward the MICROBIOTA is humoral immunity *via* secretory IgA (sIgA) [31].

Immune modulation by probiotics

There are multiple ways to study the interactions of gut microbes with the IMMUNE SYSTEM. Numerous studies have been published describing the modulation of different cells of the IMMUNE SYSTEM *in vitro*. In such studies, isolated human cells are co-cultured with microbial cells and subsequently the immune response can be characterized by analyzing cytokine production profiles (see chapter A7). These studies have revealed that microbial cells are powerful stimulants, as immune cells express specific RECEPTORS, including the TLRs, that are dedicated to recognizing microbial molecules and translating these signals into immune responses. Furthermore, the response is highly specific to the species, and even strain, that is tested. Such *in vitro* methods provide valuable screening tools and are broadly applied for mechanistic studies, but the translation of findings to effects and clinical observations *in vivo* is often challenging. In recent years, there have been a growing number of studies describing the effect of gut microbes and

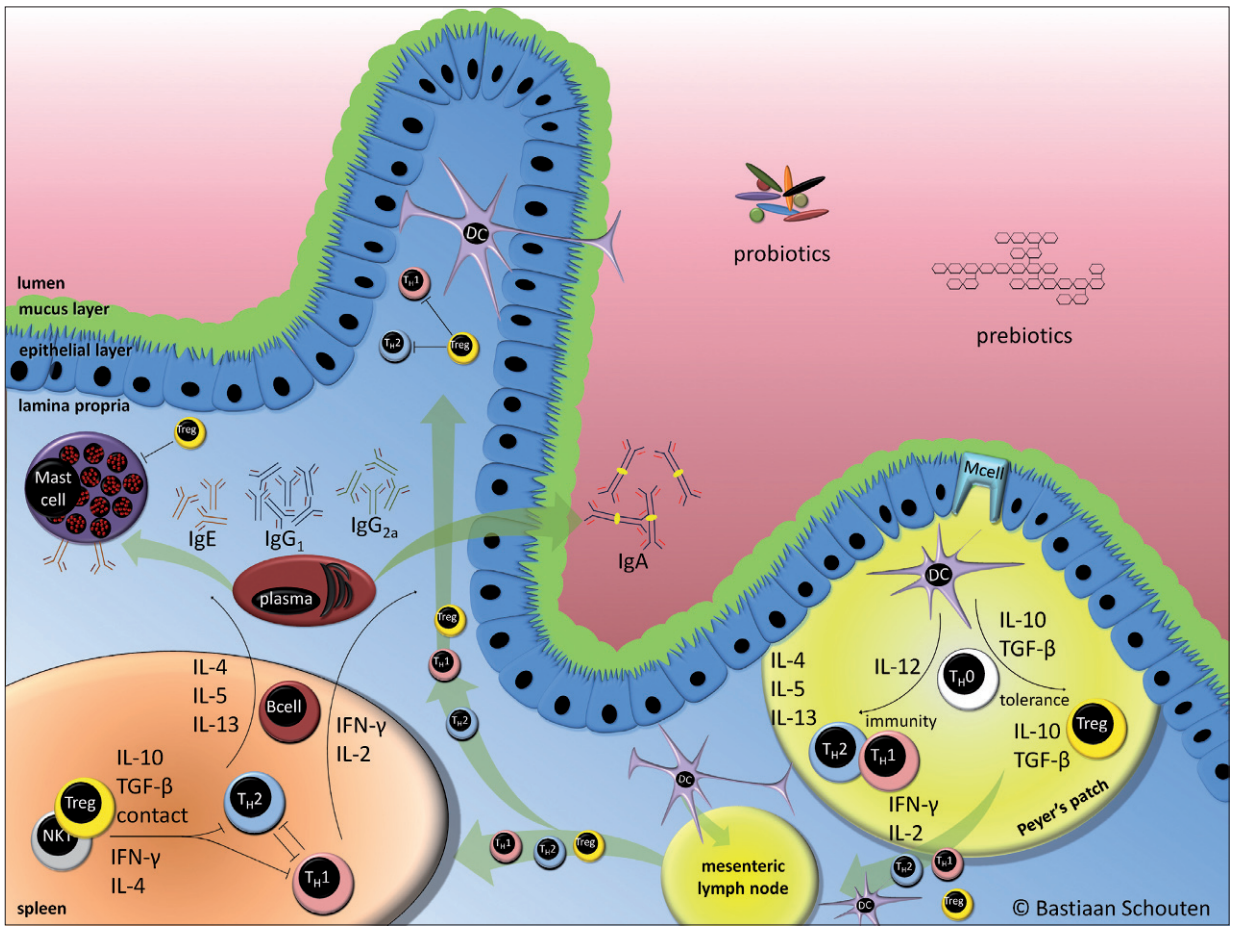

FIGURE 1. BUILDING TOLERANCE IN THE INTESTINAL MUCOSA

Food components and other substances are taken up by M-cells, after which the underlying dendritic cells (DC) in the Peyer's patch present these substances to naïve T cells (Th0), which differentiate, dependent on the surrounding cytokine milieu, into Th1 or Th2 effector cells (immunity) or into regulatory T cells (Treg) when tolerance develops. DCs can traffic from the Peyer's patch to the mesenteric lymph nodes, or sample antigen from the lumen and effector sites and then traffic to the mesenteric lymph nodes where antigens are presented to naïve T cells. Generated Treg, Th1 or Th2 cells enter the blood stream and home back to the intestinal mucosa where they become resident in the lamina propria. Treg, Th1 or Th2 cells generated in the mesenteric lymph node can also traffic to the peripheral immune system and transfer tolerance or immunity. B cells expand and mature in the spleen and traffic back to the effector site where they produce specific IgE, IgG1, IgG2a or IgA. These Igs can be transported into the lumen (IgA) or bind to mast cells (IgE, IgG1 and IgG2a) and wait for another substance exposure. This immunological cascade can be modulated by food components like prebiotics, probiotics, and the combination of these two (synbiotics). Adapted from Schouten [70].

PROBIOTICS on the mammalian and human IMMUNE SYSTEM using animal models and human subjects. Recent technological advances now allow integration of molecular analyses in such studies, providing

further evidence for the mechanisms of cross-talk between microbes and host.

As mentioned before, epithelial cells can dampen TLR signaling or expression. In addition, intestinal epithelial cells (IEC) are crucial for maintaining intestinal homeostasis [32], and failure to control inflammatory processes at the epithelial cell level may critically contribute to the disease pathogenesis. IEC react to bacterial as well as immune-derived pro-inflammatory signals by secreting CYTOKINES and CHEMOKINES, such as IL-6 and interferon-γ-induced protein 10 (IP-10, CXCL10), to attract and activate Th1 cells and phagocytic cells for defense at the site of infection. Experimental studies have confirmed that IP-10 plays a key role in uncontrolled disease development, since the blockade of IP-10 by an anti-IP-10 ANTIBODY was sufficient to decrease disease severity in IL-10 gene knockout (IL-$10^{-/-}$) mice with an IBD-like condition. This effect was due to reduced Th1 cell generation in lymph nodes and reduced recruitment of Th1 EFFECTOR CELLS to the colon [33]. A recent study showed that *Lactobacillus casei* impaired IP-10 secretion by a mechanism that did not involve impairment of initial IP-10 production, but a decrease in intracellular IP-10 protein stability. These results suggest that *L. casei* impairs vesicular pathways important for the secretion of IP-10, followed by subsequent degradation of the pro-inflammatory chemokine [34].

MACROPHAGES are another source of various CYTOKINES and large populations of MACROPHAGES are found in the normal intestinal lamina propria [35]. Direct interaction between commensal bacteria and the large number of infiltrating peripheral MONOCYTES at the onset of or during active intestinal INFLAMMATION is involved in the pathogenesis of inflammatory diseases [36, 37]. Therefore, CYTOKINES released by MACROPHAGES in response to PROBIOTICS are particularly crucial for understanding the mechanism(s) of their immunomodulatory effects on the host. Two *L. rhamnosus* strains, GG and GR-1, potently induce production of granulocyte-colony stimulating factor (G-CSF), which is a crucial mediator for suppressing TUMOR NECROSIS FACTOR (TNF) production through an activating signal transducer and activator of transcription (STAT) 3. Subsequently, activation of c-Jun-N-terminal kinases (JNKs) in MACROPHAGES is inhibited. These results define G-CSF as a key mediator through which PROBIOTICS, particularly *L. rhamnosus*, elicit immunomodulatory effects on the host [38].

Although the role of PROBIOTICS is established in some diseases, evidence of probiotic immune modulation is more difficult to obtain in the healthy population, whereas this is crucial when targeting immune benefits. In this context, the impact of daily consumption of a probiotic dairy drink containing the probiotic strain *L. casei* DN-114 001 was studied on the immune response to influenza vaccination in an elderly population in whom immune responses are known to be weaker compared to younger adults [27]. Vaccination occurred after 4 weeks of product consumption. The results showed that influenza-specific IgG1 titers for the three viral strains composing the vaccine (H1N1, H3N2, and B) increased after vaccination, being consistently higher in the probiotic product group compared to the control group. Similarly, ANTIBODY titers against the B strain increased significantly more in the probiotic group than in the control group at 3, 6 and 9 weeks post-vaccination under product consumption. These studies indicate that consumption of these PROBIOTICS increased ANTIBODY responses to influenza vaccination, which may provide a health benefit in this population.

Further investigation of the mode of action is now possible as a result of the breakthrough developments in the field of genomics, providing a sensitive tool to assess the global responses of molecular cross-talk between microbes and the host. This potential was exploited in a study by Van Baarlen et al. [39], who showed in a double-blind randomized control study with healthy adults that ingested preparations of living and heat-killed *L. plantarum* bacteria activated specific mucosal immune responses. In this intervention study, biopsies from the intestinal duodenal mucosa were taken and altered GENE EXPRESSION profiles were analyzed using whole-genome microarrays and by biological pathway reconstructions. Expression profiles of human mucosa displayed striking differences in the modulation of NF-κB-dependent pathways, notably after consumption of living *L. plantarum* bacteria in different growth phases. This is most likely due to changes in the composition of the cell wall, or physi-

ological state of the bacterium. These results provide a unique and high resolution view of the molecular interactions between microbe and host and open new avenues towards understanding the mode of action of health ingredients and PROBIOTICS.

Immunomodulatory compounds in human breast milk

The WHO refers to breast milk as the Golden Standard for baby nutrition. From a nutritional point of view, breast milk compounds are therefore interesting targets for immunomodulatory research. Several studies have shown the anti-infective properties of human breast milk, as it reduces the incidence of GI and non-enteric infections in infants [5]. This is due to its anti-microbial activity against several viruses, bacteria and protozoa. In addition, breastfeeding can reduce infant mortality, and provide protection for instance against neonatal meningitis and septicemia. Moreover, protection has been reported against respiratory infections, immunological diseases like insulin-dependent diabetes and tumors in infancy, as well as reduced development of inflammatory conditions like ALLERGY, Crohn's disease, and ulcerative colitis.

Besides these anti-infective properties, anti-inflammatory properties of breast milk have been described. This is mainly of importance during the bacterial colonization of the newborn's mucosal surfaces, including the skin and gut. Here, a huge amount of microbial components are brought in acute contact with the sterile neonate. Coordination of the inflammatory response developed after this first contact is of vital importance. The epithelial layer, together with the intraepithelial and lamina proprial immune competent cells, is the most important player in regulating the recognition of micro-organisms and maintenance of gut homeostasis. Taken together, this emphasizes the diverse activity of human breast milk compounds [5].

Breast milk contains several immunomodulatory compounds including IgG, IgM and isoforms of IMMUNOGLOBULINS (sIgA), nucleotides, specific amino acids (taurine, polyamines), polyunsaturated fatty acids (PUFAs; eicosapentaenoic acid, docosahexaenoic acid), monoglycerides, leuric acid, linoleic acid, CYTOKINES and CHEMOKINES, soluble RECEPTORS (CD14, TLR2), antibacterial proteins/peptides (lactoferrin, lysosyme, β-lactoglobulin, casein), carbohydrates and intact immune cells.

Carbohydrates

Carbohydrates in breast milk, like lactose, glycoconjugates and oligosaccharides, mainly function as important sources for energy production. Oligosaccharides are non-digestible food ingredients that modify the balance in the intestinal MICROBIOTA by stimulating the growth of beneficial bacteria like bifidobacteria and lactobacilli [1]. Since the non-digestible oligosaccharides present in human breast milk showed a clear bifidogenic effect on the gut MICROBIOTA, these oligosaccharides can be considered to be prebiotic.

Besides the indirect effects of human oligosaccharides on the IMMUNE SYSTEM through their beneficial influence on the gut MICROBIOTA, the direct effects of these oligosaccharides on immune cells cannot be excluded. In human milk, the proportion of prebiotic carbohydrates is substantial, whereas the prebiotic oligosaccharides in cow's milk are present only in trace amounts. Oligosaccharide structures that can mimic the prebiotic effect of breast milk include galacto- (GOS) and fructo- (FOS) oligosaccharides.

Galacto-oligosaccharides

Several types of GOS with different chemical characteristics have been used in research with a focus on immunomodulatory effects. GOS produced by glycosylation of lactose, using β-galactosidase enzymes, is mostly referred to as trans-GOS, β-linked GOS or TOS in the literature. GOS produced by elongation of galactose by α-galactosidases is often referred to as α-GOS [40]. The α-galactosyl derivates of sucrose occur widely in nature; the trisaccharide raffinose and the tetrasaccharide stachyose are present in soy beans and many other plants. Examples of the chemical

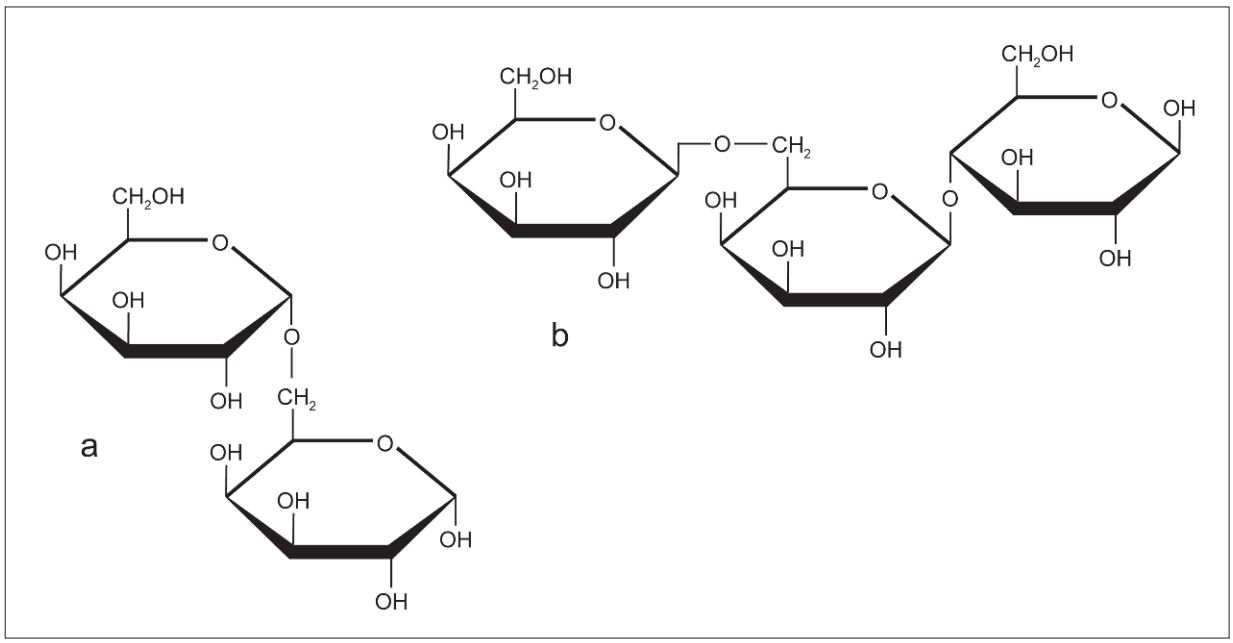

Figure 2. Examples of the chemical structure of α- and β-galacto-oligosaccharides (GOS)
Enzymatic elongation of galactose or lactose is used to produce α- or β-GOS, respectively. A disaccharide with α-(1,6)-linkage (a) is an abundantly produced component in the production of α-GOS, but various other linkages and trisaccharides and tetrasaccharides also occur [40]. β-GOS may contain a variety of chain lengths (majority is dp <6) and linkages. An example is shown with a β-(1,6)-linkage (b). Adapted from Vos et al. [71].

structure of α- and β-galacto-oligosaccharides are depicted in Figure 2.

GOS has not been studied widely with respect to immunomodulating effects as a single dietary agent. Although anti-allergic and anti-inflammatory effects were described for α-GOS [41, 42] and raffinose [42, 43], more work is needed to clarify the effects of GOS and compare these with similar effects of other oligosaccharides and non-digestible carbohydrates.

Fructo-oligosaccharides

Definitions of fructans, such as various types of FOS and inulin, vary widely in literature. Most experiments that have been described in literature were performed with unprocessed chicory inulin or fructans derived from chicory inulin. The basic structures of carbohydrate chains in fructans are depicted in Figure 3. Unprocessed chicory inulin is mainly composed of fructans with a degree of polymerization (dp) ranging from 2 to 60, ending with a terminal glucose monomer. Partially hydrolyzed inulin (short-cahin FOS, scFOS) has a typical dp range of 2 to 8, and there are more molecules that end without a terminal glucose monomer compared to inulin. Physical removal of short-chain fructans from chicory inulin (long-chain FOS, lcFOS) [44, 45] leads to a mixture of fructans with terminal glucose monomers and an approximate average dp of 22. FOS can also be produced by enzymatic elongation of sucrose (eeFOS) resulting in very short fructan molecules of dp 2–4 [46]. Besides the soluble forms, a specific insoluble form of inulin (γ-inulin: a crystallized form of dahlia-tuber derived inulin of dp >50) was shown decades ago to activate the alternative complement pathway and exhibit adjuvant activity when injected intraperitoneally [47–49].

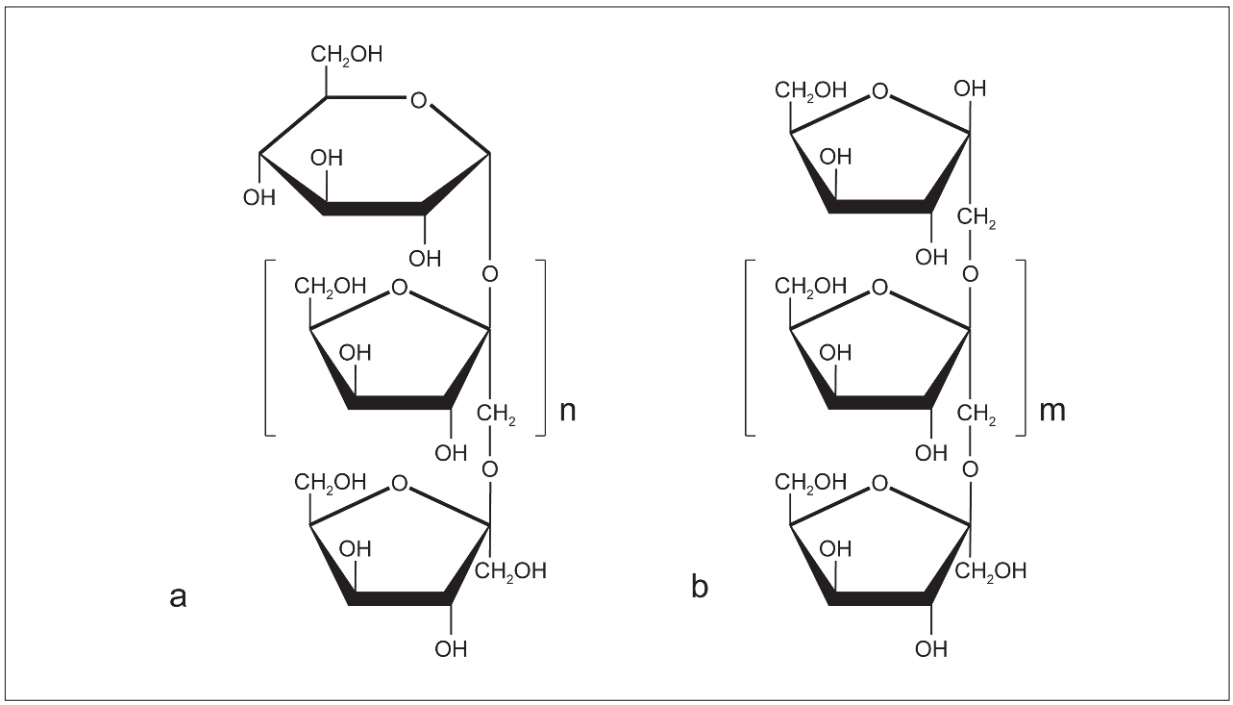

Figure 3. The basic structures of carbohydrate chains in fructans

Most fructose chains in fructan preparations end with a glucose residue at the reducing end (a). Chains that do not end with a glucose residue (b) are found in small amounts in unprocessed chicory inulin and in larger amounts in short-chain fructo-oligosaccharides (scFOS) derived from enzymatic digestion of inulin. Unprocessed chicory inulin mainly contains chains of n = 1–59. scFOS preparations are mainly composed of very small chains (n or m < 6). Adapted from Vos et al. [71].

A wide range of effects has been reported upon fructan supplementation in animal models, including anti-inflammatory effects in IBD models [50], increased survival in infection models [51], anti-carcinogenic effects, enhanced mucosal ANTIBODY responses, and modulation of mucosal cytokine patterns and cell populations [52]. Clinical studies have shown similar results in IBD patients, but other effects are not as clear. The data on infectious diarrhea and immunomodulating effects in infants and young children are interesting, but more data from well-controlled studies are needed to corroborate these findings.

Examples of immunomodulation by prebiotics in infants

The metabolic activity of the intestinal MICROBIOTA observed in breast-fed children is characterized by relatively high acetate and lactate levels and a slightly acidic pH. These physiological conditions restrict the growth of potential pathogens like *Escherichia coli* and *Clostridium perfringens*. Addition of scGOS/lcFOS to infant formula stimulated the acetate and lactate production by the MICROBIOTA and also led to a slightly acidic pH [53]. In addition, the scGOS/lcFOS formula increased the secretion of soluble IgA, which is a key factor in GI defense against dietary and microbial ANTIGENS [54]. Another study on the

effects of the scGOS/lcFOS mixture showed that the MICROBIOTA in the colon of healthy infants receiving a formula containing scGOS and lcFOS stimulated the growth of bifidobacteria and changed the metabolic activity of the total intestinal MICROBIOTA, resulting in a FERMENTATION profile that is similar to the profile found in breast-fed infants [55]. This increase in bifidobacteria is not only observed in children who start the prebiotic ingestion at 4–6 weeks, but also if infants begin the ingestion of scGOS/lcFOS when they are about to start consuming solid foods [56]. Besides an effect of scGOS/lcFOS in healthy infants, the potential of the prebiotic mixture was also investigated in infants who have a high risk for ALLERGY. It was shown that scGOS/lcFOS supplementation reduced the cumulative incidence of atopic dermatitis in the high-risk infants [57]. In the follow-up to this study, it was shown that the scGOS/lcFOS mixture reduced the total Ig response, modulating the immune response towards cow's milk proteins, whereas the response to vaccination with DTP (diphtheria, tetanus and polio) remained unaltered [58]. Several other studies, involving supplementation with a mixture of scGOS with lcFOS, showed a reduced incidence of infections and atopic dermatitis [59–61].

Examples of immunomodulation by prebiotics in HIV patients

Recently, a prebiotic intervention study was performed to investigate potential microbiological and immunological benefits among 57 HIV patients. After a 12-week intervention with a specific mixture of GOS, long-chain fructans and pectin-derived oligosaccharides in HIV-1-infected individuals not on HAART increased in bifidobacteria levels, and reduced numbers of the *Clostridium histolyticus*, *Eubacterium rectale* and *Clostridium coccoides* were recorded in the feces of the patients who ingested the GOS/FOS/ pectin-derived oligosaccharides diet [62]. The prebiotic intervention was also associated with reduced $CD4^+$ T cell activation measured as a percentage of $CD4^+/CD25^+$ T cells. In addition, improved NK cell CYTOTOXICITY was observed [63]. In this setting, oligosaccharide-based nutritional strategies could become an integral part of disease management by supporting gut integrity.

Bacterial fermented milk products

The MICROBIOTA environment is different in each individual and the potential of PREBIOTICS to confer benefits might be partially driven by the abundance of microbes that can be modulated by the administered prebiotic. In addition, person-to-person variation in the inability to absorb nutritional components may determine the EFFICACY of the compounds [64]. Pre-digestion of nutritional components by bacterial strains might be beneficial. Several studies with milk products fermented by lactic acid bacteria have been performed. In one study, heat-treated fermented infant formula strengthened the intestinal barrier and enhanced systemic immune responses to ANTIGENS without interfering with the development of oral TOLERANCE. This suggested a potential beneficial effect on host defense and vaccination [65].

Conclusions and future prospects for probiotics and prebiotics

Rapid advances in genomics and other high-throughput technologies have yielded a huge body of evidence on the importance of GI microbes in human health and the IMMUNE SYSTEM in particular. This implies that there are clear prospects for intervention to direct the activity of the IMMUNE SYSTEM using targeted modification of the composition and/or activity of the MICROBIOTA in the GI tract. This can be achieved either by ingredients influencing the GI MICROBIOTA, such as PREBIOTICS, or by directly introducing probiotic microbes with desired activity. Although many studies have shown the beneficial role of PROBIOTICS, some studies also have revealed potentially adverse effects when the products are enterally administered to critically ill patients with a low intestinal blood flow, as in a recent study in pancreatitis patients [66]. Sound pre-clinical testing and

a full toxicological profile should precede the use of specific PROBIOTICS when considering their use in any disease setting.

Even though the most well-known function of the gut MICROBIOTA is to support gut health by metabolizing dietary nutrients like oligosaccharides [1], commensals additionally actively participate in the metabolism of XENOBIOTICS [67]. Variations in drug metabolism can be attributed to inter-subject MICROBIOTA diversity as shown by the different profiles of liver and kidney metabolites in rats [68]. The concept of the gut MICROBIOTA being a potentially new pharmaceutical TARGET stems from the potential of commensals to differentially metabolize nutrients and drugs, and thus influence metabolite outcomes [69].

It can be envisioned that important progress will be made in the molecular dysbalances in the IMMUNE SYSTEM occurring during intestinal immune diseases such as IBD, and hence it will become clear how a probiotic needs to modulate the IMMUNE SYSTEM to be effective. For immune modulation in the healthy population, this will be more delicate as the desired modulation of the IMMUNE SYSTEM will largely depend on the immune status of the subject. Yet, successful clinical results illustrate the potential to bring health benefits also to the healthy population and, undoubtedly, we will gain additional insights into the underlying molecular mechanisms.

The regulatory constraints that are becoming stricter, not only in Europe, but all over the world, demand a translational research approach in which clinical results are backed-up by mechanistic studies in preclinical models. This will be crucial to build and sustain the credibility needed to fully harvest the potential of PROBIOTICS and PREBIOTICS in the years to come.

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Mild plant and dietary immunomodulators

Michael J. Parnham and Donatella Verbanac

Introduction

Plants and minerals have been used since ancient times for the treatment of many ailments and diseases. Most were used for mystical reasons and others relied on the "doctrine of signatures", which stated that the shape of the plant reflected its potential medicinal use. The root of the mandrake or ginseng, for instance, is shaped like that of the human body and has been used as a general tonic for a variety of illnesses [1]. It is claimed by herbalists to have immunostimulant properties. Siberian ginseng or Taiga root (*Eleutherococcus senticosus*) is also used as a tonic and has been reported to exhibit immunostimulatory properties. The pharmacological bases of these actions are unclear, so these plant medicines cannot be considered unequivocally as immunostimulants.

In recent years, many folklore remedies have been subjected to intensive pharmacological study and some have been shown to exhibit therapeutic immunomodulatory properties in experimental and clinical studies.

Antioxidant dietary constituents also have been shown to exert immunoprotective and/or immunostimulant properties and are widely sold as prophylactic nutritional supplements. Some of the compounds for which clear immunomodulatory actions have been described are discussed in this chapter. Combination products are not considered, since little scientific basis is available for their EFFICACY.

Plant immunostimulants

Purple coneflower (*Echinacea*)

History

The PURPLE CONEFLOWER (Fig. 1) is indigenous to North America and was used by the American Indians of the Great Plains as a universal remedy, particularly for colds, sore throats and pain [2]. Extracts of *E. angustifolia* (narrow-leaved PURPLE CONEFLOWER) were introduced into medical practice in the United States at the end of the 19th century, becoming the most widely used medicinal plants by the 1930s. With the introduction of antibiotics, *Echinacea* fell into disuse. In Europe, *E. angustifolia* was introduced into homeopathic practice in response to publications from the United States. In 1937, a general lack of supplies subsequently led to the introduction of the common PURPLE CONEFLOWER (*E. purpurea*) to Germany, where the squeezed sap of the aerial parts of the plant was marketed. Many of the pharmacological studies on *Echinacea* have been performed on this preparation in Germany.

Chemical constituents

Compounds isolated from *Echinacea* species include caffeic derivatives, FLAVONOIDS, ethereal oils, polyacetylenes, ALKYLAMIDES, alkaloids and polysaccharides [3]. Ingredients thought to contribute to the immunostimulatory properties of *Echinacea* include cichoric acid, polysaccharides and ALKYLAMIDES, the main lipophilic constituents (Fig. 2). The ALKYLAMIDES, which exhibit structural similarities to anandamide, the endogenous LIGAND of cannabinoid RECEPTORS, are the most potent stimulators of INNATE IMMUNITY [4]. Commercial preparations of *Echinacea* contain

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Figure 1. Purple coneflower (*Echinacea purpurea*)

60–80 g squeezed sap per 100 g, but the relative proportions of the various constituents vary markedly between different products. Because of this lack of uniformity, standardisation on the basis of agreed active ingredients is clearly needed.

Modes of action and pharmacological effects

The squeezed sap of *E. purpurea* stimulates the phagocytic activity of neutrophils and macrophages *in vitro* and *in vivo*. The response is moderate, but a significant increase in neutrophil phagocytosis has been observed following repeated oral administration to healthy volunteers [3]. With many phytopharmaceuticals, pharmacological effects are thought to be due to a combination of constituents. Stimulation of macrophage phagocytosis appears to be most pronounced with the alkylamides (particularly dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamides) present in the *E. purpurea* preparation [4]. In contrast, when tested on bacterial lipopolysaccharide (LPS)-stimulated macrophages, these alkylamides weakly reduce cyclooxygenase (COX) activity and inhibit the expression of TNF-α, the latter effect (together with reduced IL-8) also being observed after oral administration of *Echinacea*. The release of TNF-α is blocked by antagonists of cannabinoid CB2 receptors, for which the alkylamides have affinity fivefold less than that of anandamide [4]. Consequently, both stimulation of phagocytosis and CB2 receptor-mediated inhibition of inflammatory cytokines appear to contribute to the therapeutic response to *Echinacea*. Other actions, such as stimulation of natural killer (NK) cell activity, modification of circulating leukocyte populations, possible effects on T cell-derived cytokines and antibody formation, as well as direct antiviral actions of alkylamides also probably contribute to the oral activity of the preparation [4, 5]. In addition, cynarin (1,3-dicaffeoylquinic acid), a minor component of *Echinacea purpurea* extract, is a weak but selective inhibitor of CD28-mediated signal transduction in activated T cells [6]. Administered topically to the skin, the squeezed sap of *E. purpurea* enhances wound healing, probably by inhibiting hyaluronidase leading to increased hyaluronic acid secretion.

Cellular pharmacokinetics

The 2, 4-diene alkylamides are transported, without significant metabolism, across monolayers of CaCo-2 colonic epithelial cells and dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamides are detectable in human blood after oral administration of *E. purpurea* extract [4, 7, 8]. The t_{max} of the alkylamides is around 30 minutes after oral *Echinacea* administration, but this is delayed by 10–2 hours at higher concentrations of extract, possibly due to micelle formation by the lipophilic alkylamides. Degradation of alkylamides occurs by hepatic oxidative metabolism.

Clinical indications

Non-homeopathic preparations of *E. purpurea* are used mainly for the oral adjuvant treatment of respi-

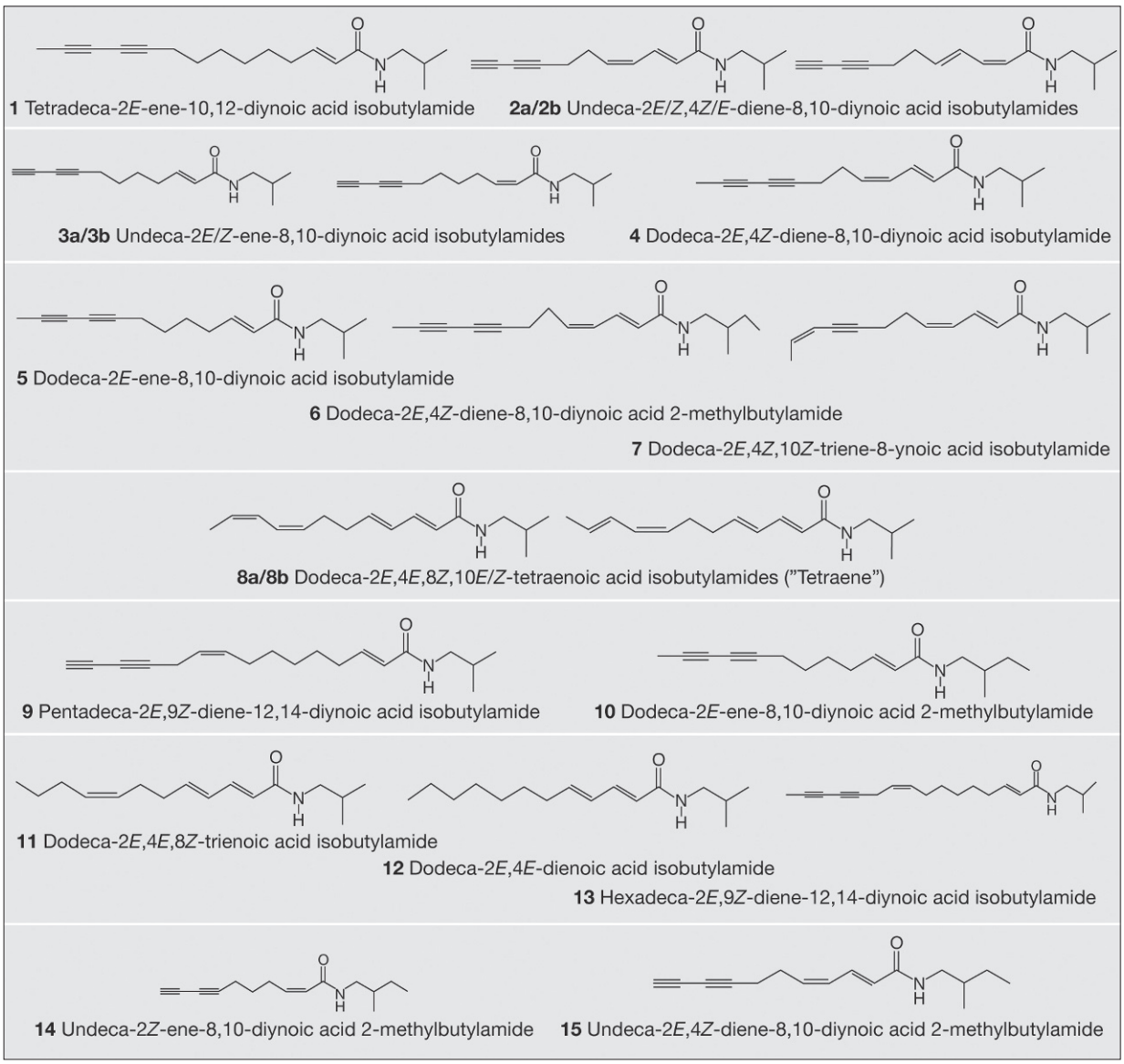

Figure 2. Structures of the main *Echinacea* alkylamides (from [4] with permission).

ratory and urinary tract infections and also topically for wound healing. Several double-blind, controlled clinical trials have confirmed moderate efficacy in the treatment of mild respiratory infections [9]. When administered within a few hours of symptoms arising, *E. purpurea* has been reported to shorten the course of the common cold, but without reducing symptom intensity [10]. However, while *Echinacea* use for respiratory indications remains extensive, negative outcomes in some studies, possibly due to insufficiently standardised product, mean that evidence for efficacy remains inconclusive [11]. Efficacy in the treatment of vaginal candidiasis has only been reported in open studies.

Side effects

Common adverse reactions reported with *Echinacea* include headache, dizziness, nausea, constipation and mild epigastric pain. No adverse effects that are specific to *E. purpurea* have been observed, but as with all plant extracts, HYPERSENSITIVITY responses (dermatitis, ANAPHYLAXIS) have been reported, which in rare cases can be severe. In the US, because of the occasional occurrence of skin rash, *Echinacea* preparations are not recommended for use in children. In Germany, on the basis of theoretical considerations, *Echinacea* products are to be avoided in patients with autoimmune disorders.

Mistletoe (*Viscum album*)

History

Extract of the leaves (not berries) of mistletoe (Fig. 3) has been used for centuries in Europe as a traditional herbal treatment for infections. In the last century, Rudolf Steiner, the originator of ANTHROPOSOPHY, suggested its use as a remedy for cancer. Biochemical analysis of mistletoe constituents led, in the 1980s, to the isolation and characterisation of specific CYTOTOXIC LECTINS that are responsible for the proposed antitumour activity of the extract [12]. The herb is not approved for use in the United States.

Chemical constituents

The main immunostimulatory constituents of mistletoe are the glycosylated LECTINS, ML-I, ML-II and ML-III. The major component is ML-I (viscumin), a member of the type II ribosome-inactivating proteins, which is used to standardise mistletoe extracts. It consists of two polypeptide chains linked by a disulphide bridge. The A-chain has enzymatic rRNA-cleaving activity and the B-chain binds to the TARGET cell. Other constituents of mistletoe include FLAVONOIDS, viscotoxins, terpenoids and polysaccharides. Amines, such as ACETYLCHOLINE, HISTAMINE and tyramine are also present and may contribute to hypotensive effects of mistletoe preparations.

FIGURE 3. MISTLETOE (*VISCUM ALBUM*)

Modes of action and pharmacological effects

ML-I has a broad range of affinities for α/β-linked galactopyranosyl residues. High nanogram concentrations of all three mistletoe LECTINS are CYTOTOXIC. This action is due to ribosome inactivation by the rRNA *N*-glycosidase A-chain [13], leading to induction of APOPTOSIS, possibly through activation of cation channels. At lower concentrations, ML-I and ML-I-standardised mistletoe extracts stimulate release of IL-1, IL-6 and TNF-α from peripheral blood mononuclear and skin cells [14]. Repeated doses of mistletoe extract at an ML-I-equivalent of 1 ng/kg s.c. in cancer patients cause an increase in body temperature, increases in circulating Th cells and NK cells and enhanced expression of IL-2 RECEPTORS (CD25) on LYMPHOCYTES [15]. A direct stimulatory action on T cells is likely. In mice, mistletoe extract reduces formation of melanoma metastases. Direct CYTOTOXIC effects of the extract, on injection into gynaecological tumours in mice, have been observed, but on systemic administration, inactivation by serum glycoproteins

and anti-ML ANTIBODIES occurs [16]. Mistletoe extract is inactive on oral administration and only exhibits immunostimulatory activity on parenteral injection, possibly through activation of MACROPHAGES.

Cellular pharmacokinetics

Using gold-labelled ML-I, the LECTIN has been shown to be taken up into L1210 leukaemia cells *in vitro via* coated pits and *via* plasma membrane endocytosis. These kinetics correlate with the CYTOTOXICITY of ML-I both with regard to time and concentration [17].

Clinical indication

In German-speaking countries, ML-I-standardised mistletoe extract is administered intracutaneously at increasing dosages from 0.5 to 1.0 ng/kg twice weekly for at least 3 months in cancer patients. It has been used in patients with breast, uterine, cervical, vulvular and ovarian tumours [16]. A recent Cochrane Review evaluated the results of 21 prospective, randomised clinical trials of mistletoe treatment [18]. In 6 trials, improvement in survival was reported, but none of the studies were considered to be of high methodological quality. No incontrovertible evidence for tumour remission has been obtained as yet. Two studies in breast cancer patients showed clear benefit of mistletoe treatment on quality of life (QOL), but further high-quality studies are required to confirm this and other potential benefits of the therapy. Case reports of improved QOL in patients with hepatitis C have also appeared, but also require confirmation in controlled, randomised trials. Non-glycosylated RECOMBINANT ML-I (rViscumin) has been shown to exhibit CYTOTOXICITY in animal tumour models and is under investigation in cancer patients by intravenous infusion [19].

Side effects

In all clinical studies, mistletoe extract has been shown to be well tolerated at therapeutic doses. The extract can cause fever, headache, leukocytosis, orthostatic hypotension, bradycardia, diarrhoea and HYPERSENSITIVITY reactions. Toxic doses may cause coma, seizures and death.

Zinc

History

Zinc (Zn) is an essential mineral that is found in almost every cell. Its importance for human health was first documented in 1963. Zinc is required for many biological functions [20]. It stimulates the activity of approximately 100 enzymes, promoting various biochemical reactions in the body. Zinc is needed for DNA synthesis, proper immune response, wound healing and helps maintain sense of taste and smell. Furthermore, zinc supports normal growth and development during pregnancy, childhood, and adolescence. The element is found in almost all food, but the majority of zinc in the diet is provided by sea food, red meat and poultry. Other good food sources are beans, nuts, whole grains, fortified breakfast cereals, and dairy products [21]. Zinc absorption is greater from a diet high in animal protein than a diet rich in plant proteins, because of the presence of PHYTATES, which are found in whole grain cereals and legumes and can interfere with zinc absorption.

Recommendations for adequate dietary zinc intake by humans in the US and Canada have been revised recently, and a summary is given in Table 1.

Pharmacology

Over the past 25 years, deficiency of zinc in humans due to nutritional factors and several disease states has been gradually recognised. Alcoholism, malabsorption, sickle cell anaemia, chronic renal disease, and chronically debilitating diseases are known to be predisposing factors for zinc deficiency. Vegetarians may need as much as 50% more zinc than non-vegetarians because of the lower absorption of zinc from plant foods [21]. Individuals who have had gastrointestinal surgery or who have digestive disorders that result in malabsorption, including sprue, Crohn's disease and short bowel syndrome are also at greater risk of a zinc deficiency.

The IMMUNE SYSTEM is adversely affected by even moderate degrees of zinc deficiency, while severe zinc deficiency depresses immune function [22]. Zinc is unequivocally important for innate, as well

Table 1. Daily recommended intakes for zinc

		Recommended dietary allowance for zinc	
Life stage[a]	**Age**	**Males (mg/day)**	**Females (mg/day)**
Infants	0–6 months	2	2
Infants	7–12 months	3	3
Children	1–3 years	3	3
Children	4–8 years	5	5
Children	9–13 years	8	8
Adolescents	14–18 years	11	9
Adults	19 years and older	11	8
Pregnancy	18 years and younger	–	12
Pregnancy	19 years and older	–	11
Breast-feeding	18 years and younger	–	13
Breast-feeding	19 years and older	–	12

[a]*Source [21]; Updated in February 2008 by Victoria J. Drake, Ph.D. Linus Pauling Institute Oregon State University; Reviewed in February 2008 by: Emily Ho, Ph.D. Associate Professor of Nutrition and Exercise Sciences Principal Investigator, Linus Pauling Institute Oregon State University*

as for the adaptive immune response. Decreased zinc concentrations impair NK cell activity, neutrophil and macrophage PHAGOCYTOSIS, CHEMOTAXIS and oxidative burst generation [23]. The element is required for recognition by KILLER CELL INHIBITORY RECEPTORS (KIR) expressed on NK cells of MHC class I molecules (predominantly HLA-C) on TARGET cells. In this way, zinc can influence NK cell-mediated killing of virus-infected and tumour cells and modulate the action of cytolytic T LYMPHOCYTES. In addition, zinc is required for the development and activation of T LYMPHOCYTES [21]. The element is an essential cofactor in stabilising thymulin, the thymic hormone that is a key factor for differentiation and maturation of immature T LYMPHOCYTES in the thymus and in the periphery. Thymulin acts on cytokine secretion by peripheral blood mononuclear cells and, together with IL-2, induces proliferation of $CD8^+$ T cells. Zinc also influences mature T cells, inducing the expression of the high-AFFINITY RECEPTOR (CD25) for IL-2. This could be the reason why decreased T cell proliferation and anergy is observed after MITOGEN stimulation during zinc deficiency. Another possible reason could be that zinc is essential for binding of the protein tyrosine kinase p56 (Lck) to the α chains of the T cell co-RECEPTORS, CD4 and CD8, a signalling step necessary for T LYMPHOCYTE development and activation. Association of p56 (Lck) with CD4 requires two conserved cysteine residues in the cytosolic domain of CD4 and two in the N terminus of p56(Lck), and zinc is essential for this complex formation [24].

It has also been known for more than 25 years that zinc ions can induce blast transformation in human LYMPHOCYTES. Therefore, zinc can be considered the simplest MITOGEN known. When added to peripheral blood mononuclear cells at stimulatory concentrations, it induces the release of IL-1, IL-6, TNF-α and IFN-γ, an effect independent of the presence of LYMPHOCYTES. In contrast to this direct stimulation of MONOCYTES, the stimulatory effect on T cells is indirect and strictly dependent on the presence of MONOCYTES in the cell culture.

When zinc supplements are given to individuals with low zinc levels, the numbers of circulating T cells increase and the ability of LYMPHOCYTES to fight viral and bacterial infection improves. For instance, malnourished children in India, Africa, South America, and Southeast Asia have been shown to experience shorter courses of infectious diarrhoea after taking zinc supplements [25]. The importance of zinc supplementation during aging has been recently recognised and deserves special attention (see Box 1).

Cellular pharmacokinetics

Zinc supplements are available in oral and parenteral formulations. Available oral formulations include zinc sulphate, zinc gluconate, zinc picolinate and the newest form of supplementary zinc, zinc monomethionine, while zinc chloride and zinc sulphate are available as injections. Zinc monomethionine is the most bioavailable form of zinc, because the molecule is transported through small intestinal epithelial cells using the endogenous transport system for methionine. Foods containing high amounts of phosphorus, calcium, or PHYTATES (found in bran, brown bread) can reduce the amount of zinc absorbed. The same effect has been observed with caffeine-rich beverages and food. Commonly used sweeteners, such as sorbitol, mannitol, and citric acid make zinc lozenges ineffective. Drug–zinc interactions have been observed with quinolone and tetracycline antibiotics and PENICILLAMINE. It is recommended, therefore, to take zinc 6 hours before or 2 hours after antibiotics.

Clinical indications

Zinc is an essential trace element used to treat zinc deficiency and delayed wound healing associated with zinc deficiency. It is also used for herpes simplex, Hansen's disease, diabetes, dental plaque, Alzheimer's disease, Wilson's disease, colds, acne and other skin problems, and to stimulate the IMMUNE SYSTEM to fight infection. With WHO support, inexpensive zinc tablets are being widely distributed in poor areas of Asia and Africa in a successful drive to combat fatal diarrhoea in young children, resulting from inadequate sanitation.

Side effects

Rare side effects with large (<150 mg/day) doses of zinc include chills, sustained ulcers or sores in the mouth or throat, fever, heartburn, indigestion, nausea, sore throat, unusual tiredness or weakness. The symptoms of overdose (>150 mg/day) include chest

Box 1. Interrelationship between zinc and immune functions during aging

In addition to other nutritional factors reported to enhance innate immunity in the elderly, the trace element zinc plays a pivotal role in sustaining NK cell cytotoxicity. Zinc (10 μM) improves development *in vitro* of $CD34^+$ cell progenitors to NK cells from both young and older donors. Since zinc turnover is mediated by METALLOTHIONEINS (MTs), these proteins are key to understanding the role of NK cells in aging and longevity. The involvement of zinc and MTs in immune function during the regeneration process was first reported in the late 1990s in the model of liver regeneration in young and old mice [26]. Partial hepatectomy and aging both caused a significant increase in MTs, which was associated with low availability of free zinc for uptake into thymocytes and extrathymic T cells. To avoid this effect of MTs on zinc availability in the elderly, supplementation with zinc may be useful to improve immune responses in old age. However, this supplementation should be performed with caution to avoid possible competition with copper [27].

MT homeostasis and intracellular zinc bioavailability is crucial in attenuating the link between increasing age and chronic inflammation, which is becoming increasingly apparent [28]. Deterioration of innate immune function and the worsening of the chronic inflammatory status can lead to the development of common age-related pathologies such as type 2 diabetes or cardiovascular diseases. In contrast, maintenance of the balance between zinc, MT and innate immunity may help elderly subjects to stay healthy to a very old age [29].

pain, dizziness, fainting, shortness of breath, vomiting, yellow eyes or skin. Cases of zinc toxicity have been seen in both acute and chronic forms. Intakes of 150–450 mg zinc per day may be accompanied by low copper status, altered iron function, reduced immune function, and reduced levels of high-density lipoproteins [30].

Dietary antioxidants

Phagocytic cells, including GRANULOCYTES and MACROPHAGES, generate large quantities of REACTIVE OXYGEN SPECIES (ROS) following activation. This makes the lipid membranes of cells of the IMMUNE SYSTEM particularly susceptible to oxidative damage with subsequent IMMUNOSUPPRESSION. Several constituents of the normal diet help to protect against the damaging effects on lipid membranes of ROS, such as SUPEROXIDE ANION, H_2O_2 and hydroxyl radical, which are formed during a variety of physiological oxidation processes. Vitamin E is a lipid-soluble antioxidant that breaks the chain reaction of lipid peroxidation by scavenging peroxy radicals. The resulting vitamin E radical is transferred to water soluble antioxidants, such as vitamin C (ascorbate), for excretion in the urine. Selenium is an essential dietary trace element and is incorporated into the active site of the enzyme GLUTATHIONE PEROXIDASE (GPx). GPx catalyses the breakdown of hydroperoxides to hydroxy acid (oxidising GSH to GSSG), thereby complementing the action of vitamin E (Fig. 4). Among these nutrients, vitamins C and E and selenium have been shown to have clear immunostimulant/immunoprotective properties and play a role in disease prophylaxis.

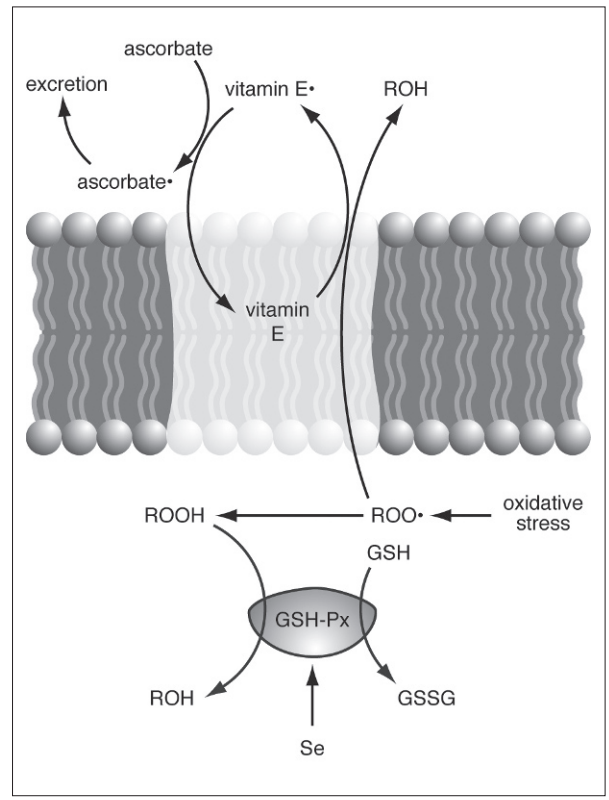

FIGURE 4. INTERPLAY BETWEEN SELENIUM, VITAMIN E AND VITAMIN C IN PROTECTION OF MEMBRANES FROM OXIDATIVE DAMAGE
ROOH, lipid hydroperoxide; ROO˙ ,hydroperoxy radical; ascorbate˙, semidehydroascorbate radical; vitamin E˙, vitamin E radical

Selenium

History

The element selenium (Se) was discovered in 1818 by the Swedish chemist Berzelius, who named it after Selene, the Greek goddess of the moon. A biological role for selenium was first demonstrated in 1957 by Klaus Schwarz who found that selenium protected against dietary liver degeneration in rats. Subsequently, in 1973, Flohé in Germany and Rotruck in the United States showed that selenium is present at the active site of the enzyme GPx, where it is incorporated as selenocysteine. Since then, 24 new SELENOPROTEINS have been identified in humans and characterised.

Pharmacology

Dietary selenium is important for a healthy immune response. There is also evidence that selenium has a protective effect against some forms of cancer, that it

may enhance male fertility, decrease cardiovascular disease mortality, and regulate inflammatory mediators in ASTHMA.

Incorporated as selenocysteine, the pharmacological activity of selenium is mainly expressed through physiologically important enzymes [31]. GLUTATHIONE PEROXIDASE enzymes (cellular GPx-1, gastrointestinal GPx-2, plasma GPx-3, phospholipid hydroperoxide GPx-4) represent a major class of functionally important SELENOPROTEINS, GPx-1 being the most sensitive to changes in dietary selenium. GPx-2 appears to provide mucosal defence against dietary pro-oxidants and is much more resistant to dietary selenium deficiency. GPx-3 regulates extracellular oxidant levels, including that of endogenously generated NO. GPx-4 provides protection against membrane lipid peroxidation and is involved in lipid metabolism, including that of arachidonic acid. It is also an important structural protein in the mitochondrial sheath of spermatozoa.

Thioredoxin reductases (Txnrd) are widely distributed selenocysteine containing enzymes that catalyze the NADPH-dependent reduction of thioredoxin to its oxidised form (Trx). Three mammalian forms have been described: cytoplasmic/nuclear Txnrd1 (also called TR1 or TrxR1) that reduces Trx1, mitochondrial Txnrd2 (also called TR3 or TxnR2) that reduces Trx2, and testes-specific thioredoxin–glutathione reductase (also called Txnrd3, TR2, TxnR3, or TGR). Txnrd1 and 2 are housekeeping enzymes involved in a variety of cell-signalling processes. SELENOPROTEIN M and Sep15 may be additional members of the Txnrd family.

A third major class of SELENOPROTEINS is represented by the three types of iodothyronine deiodinase enzymes (D1, D2, D3), which catalyse the 5',5-monodeiodination of the pro-hormone thyroxine (T4) to the active thyroid hormone 3,3',5-triiodothyronine (T3). D2 is believed to generate T3 from T4 locally in specific tissues including pituitary, brown fat, and brain, whereas D1 generates T3 from T4 in the thyroid and peripheral tissues primarily for export to plasma.

About 60% of Se in plasma is incorporated into SELENOPROTEIN P (Sel P), which serves as a transport protein for Se. Uptake of Sel P into tissues is mediated by binding to lipoprotein RECEPTORS, including apolipoprotein E RECEPTOR-2. Since it is expressed in many tissues, functions other than just transport are considered likely [31]. Selenium intake also affects tissue concentrations of SELENOPROTEIN W, which is reported to be necessary for muscle metabolism [32].

Diet-induced selenium deficiency is associated with a variety of defects in neutrophil and lymphocyte functions in experimental and domestic animals, which can be reversed by selenium supplementation [33]. These defects are considered to be due to a reduction in the activity of protective GPx in association with increased production of ROS, such as that occurring during the oxidative burst of PHAGOCYTES. As a result, cells in the vicinity of actively phagocytosing cells are damaged. This process also occurs to some extent in selenium-adequate animals, in which GPx-1 activity (which is very sensitive to local changes in oxidative stress) decreases in cells at local sites of acute INFLAMMATION. Circulating levels of Sel P are reduced in several chronic inflammatory diseases and it appears to act like GPX-1 in MACROPHAGES to protect them from auto-oxidative damage during parasite CLEARANCE [34]. In NEUTROPHILS from humans with a low selenium status, addition of sodium selenite *in vitro* is able to enhance the phagocytic and bactericidal activities of the cells [35], probably by protecting them from autolytic damage.

In addition to protecting PHAGOCYTES from damage, inorganic selenium administered to animals in nutritional excess has been shown to enhance ANTIBODY titres in response to vaccines or sensitisation to ERYTHROCYTES. Studies on human LYMPHOCYTES *in vitro* suggest that sodium selenite selectively enhances the synthesis of IgG ANTIBODIES [36]. Enhancement of CYTOTOXIC lymphocyte activity is a consistent response to selenium supplementation of animals and humans in nutritional excess, with increased expression of IL-2 RECEPTORS on peripheral T cells [33]. Administered to patients on haemodialysis, selenium supplementation (200–500 μg, three times weekly) enhanced T cell responses to mitogens as well as delayed HYPERSENSITIVITY responses [37]. A similar enhancement of Th1 cell responses (increased IFN-γ and IL-10 production and circulating CD4 counts, more rapid T cell proliferation) to oral live attenuated poliomyelitis vaccine has been reported

in human subjects receiving selenium supplementation. A more rapid CLEARANCE of the poliovirus was observed in the supplemented subjects [38]. However, the effective dose range for selenium supplementation above nutritional requirements is relatively narrow, since increasing the dose leads to IMMUNOSUPPRESSION.

Cellular pharmacokinetics

Cellular uptake of selenium, GPx activities, and cytoprotection has been compared in human hepatoma cells (HepG2). Selenite and selenocysteine serve as Se donors with high BIOAVAILABILITY. In contrast, selenium from selenomethionine is usually incorporated into cellular proteins but has no effect on GPx activities or cytoprotection. Consequently, not all donor forms of selenium provide selenium in a bioactive form to act as an antioxidant. Cellular selenium content, in general, does not correlate with the cytoprotective activity of this trace element, in contrast to cellular GPx activities, which always correlate, irrespective of the Se donor, with protection against lipid hydroperoxides. Thus, cellular GPx represents a more reliable marker of adequate Se supply [39].

After injection of radiolabelled Se, the metabolic turnover of Sel P in plasma peaks at 6–9 hours, whereas that of extracellular GLUTATHIONE PEROXIDASE (eGPx) is sustained for at least 24 h. Selenium is rapidly incorporated into hepatic Sel P in the liver, followed by slow and steady incorporation into renal eGPx [40]. Oral selenium (as ^{77}Se in seleno-yeast) is also rapidly absorbed with a plasma peak at 9 hours and is retained in the body for several weeks [41].

Clinical indications

Sodium selenite or seleno-yeast is widely available as a nutritional supplement, providing 50–100 μg selenium/day. This is of benefit immunologically in subjects with inadequate selenium intake, including patients on total parenteral nutrition. Keshan's disease is a cardiomyopathy associated with coxsackie virus (CVB3) in areas of China with endemic soil selenium deficiency. Selenium supplementation completely prevents the disease, at least partially by promoting the development of immunity to the virus. Several chronic viral infections, including that to HIV-1 are associated with low selenium levels and selenium supplementation has been shown to enhance NK activity and CD4 T counts in HIV-1-infected patients, though the clinical benefit is unclear [42]. Although serum selenium status is low in various inflammatory skin diseases and RHEUMATOID ARTHRITIS, clear therapeutic benefit of nutritional supplementation with selenium has yet to be demonstrated. There is even some suggestion that the tendency for selenium supplementation to promote preferentially Th1 cell-mediated responses may lead to inhibition of Th2-mediated responses in ASTHMA [42]. Like vitamin E, selenium supplementation enhances lymphocyte proliferation responses in the elderly, but there is still no clear consensus on the potentially beneficial effects of selenium supplementation on immune function in the aged population [42, 43]. There is growing evidence that prolonged selenium intake in nutritional excess is associated with a reduced incidence of a variety of cancers. Because many geographical areas – particularly Finland, parts of China, New Zealand and the UK – have low soil selenium content, nutritional supplementation with sodium selenite or selenium-enriched yeast is widespread.

Side effects

Selenium as sodium selenite or selenomethionine is considered to be non-toxic on repeated ingestion up to approximately 1000 μg Se per day. Above this dose, hair and nail loss and skin lesions can arise. At higher intakes, nervous system abnormalities, including numbness, convulsions and paralysis occur.

Vitamin C (ascorbic acid)

History

The great seafaring voyages of the Middle Ages meant that sailors were at sea for many months on very poor food rations. Many suffered exhaustion and depression, bleeding gums, haemorrhaging and bruising with fatal diarrhoea, lung and kidney damage – the symptoms of SCURVY. In 1747, the British

physician, J. Lind, found that two oranges and one lemon a day could relieve the symptoms of SCURVY, but it was not until 1795 that the Royal Navy decreed that all sailors should be given regular lime juice. The "scorbutic principle" was only identified after 1928, the year in which Albert Szent-Gyorgi isolated hexuronic acid as the factor that prevented browning of decaying fruit. The name was changed to vitamin C following structural identification and to ascorbic acid in recognition of its ability to prevent SCURVY. Szent-Gyorgi received the Nobel Prize for Physiology and Medicine in 1937. Vitamin C is now known to be a co-factor for a variety of physiological hydroxylation reactions, including those involved in catecholamine and carnitine synthesis and that of proline during collagen synthesis. It is also a co-factor for the biosynthesis of drug-oxidising cytochrome P450.

Pharmacology

Vitamin C is present at high concentrations in NEUTROPHILS and is required for optimal PHAGOCYTOSIS [44]. During vitamin C deficiency (SCURVY) almost every component of the IMMUNE SYSTEM is compromised [45]. This is mainly due to the fact that vitamin C, being a water soluble antioxidant, is able to scavenge free radicals in the extracellular compartments, which is the prime antioxidant defence in plasma. Lack of vitamin C opens circulating white blood cells to radical attack with subsequent membrane damage and suppression of cell function. Vitamin C *in vitro* inhibits activation of NF-κB, the transcription factor for cytokine expression and inhibits T cell APOPTOSIS [46, 47]. The extent to which vitamin C, at doses above the dietary requirement, is able to further enhance immune responses is still unclear. Many studies have been confounded by the administration of additional antioxidants, which are required for the full protective antioxidant effect (Fig. 3).

Cellular pharmacokinetics

Human LEUKOCYTES (NEUTROPHILS, MONOCYTES and LYMPHOCYTES) take up the oxidised form of ascorbic acid, dehydroascorbic acid, actively *via* glucose transporters, resulting in intracellular concentrations 10–100-fold higher than those in plasma. This uptake is facilitated by stimulation of the cells. Oxidation of ascorbic acid by SUPEROXIDE ANION, generated by HL-60 NEUTROPHILS undergoing an oxidative burst, leads to enhanced dehydroascorbic acid uptake by all cells in the vicinity and its immediate reconversion to ascorbic acid intracellularly [48]. This provides a feedback mechanism to enhance intracellular levels of protective vitamin C in activated LEUKOCYTES that are generating large amounts of oxygen radicals.

Clinical indications

Supplementation of subjects deficient in vitamin C (e.g. some poorly nourished elderly persons) clearly restores deficient immune responses and adequate dietary intake of vitamin C is required to sustain immune responses to infections. However, despite data from a number of clinical trials, there is little unequivocal evidence that mega-doses (<1 g/day) of vitamin C alone are able to stimulate immune responses or increase resistance to the common cold in healthy individuals [49]. Neutrophil responses, however, do appear to be enhanced in healthy subjects and children supplemented with high doses of vitamin C [49].

Side effects

In allergic persons even small amounts of vitamin C (50 mg) may cause breathing problems, tightness in the throat or chest, chest pain, skin hives, rash, itchy or swollen skin. Taking large amounts (in grams) may cause diarrhoea.

Vitamin E

History

Vitamin E was discovered in 1922 by H. Evans and K. Bishop as a dietary factor required for normal rat reproduction. It was officially recognised only in 1968. Vitamin E is a generic description for all tocol and tocotrienol derivatives exhibiting the biological activity of α-tocopherol. Together with vitamin C

and selenium, it contributes to the protection of cell membranes against oxidative damage (Fig. 3).

Pharmacology

LYMPHOCYTES and mononuclear cells have the highest vitamin E content of any cells in the body. Exposure of these cells to oxidative stress, such as that which occurs during INFLAMMATION or infection, leads to a loss of vitamin E, damage to cell membranes and cellular dysfunction. Addition of vitamin E *in vitro* to LYMPHOCYTES that have been subjected to lipid peroxidation reverses IMMUNOSUPPRESSION, measured in terms of cell proliferation and ANTIBODY formation. This protective action of vitamin E is seen most clearly in experimental vitamin E deficiency in animals. Under these conditions, ANTIBODY titres and ANTIBODY-forming cells are severely depressed, T cell responses, including proliferation and IL-2 production, are decreased and mortality to various infections is enhanced [49]. In all cases, supplementation with vitamin E reverses the IMMUNOSUPPRESSION. Prolonged vitamin E supplementation of mice also partially reverses IMMUNOSUPPRESSION caused by retrovirus infection [50].

Cellular pharmacokinetics

Vitamin E is highly lipid soluble and therefore rapidly absorbed after oral ingestion and incorporated into cell membranes.

Clinical indications

Vitamin E supplements are available for the treatment of deficiency symptoms and for the protection of muscles, blood vessels and the IMMUNE SYSTEM from the effects of oxidation. Vitamin E deficiency in humans is rare, but can arise in preterm infants, in association with impaired neutrophil phagocytic capacity. Phagocytic activity can be restored by administration of vitamin E to newborn children including those with glutathione deficiency [51].

The most convincing indication for clinical supplementation with vitamin E to achieve immunostimulation is the aging subject. The activities of antioxidant enzymes decrease with age, leading to a general increase in lipid peroxide tone in the body. In elderly people, nutrition can be sub-optimal and in such subjects supplementation with vitamin E has been shown to increase DTH skin test responses, enhance ANTIBODY responses to vaccines as well as MITOGEN-induced lymphocyte proliferation and IL-2 production [49, 52, 53]. Whether this enhancement of immune responses by prophylactic vitamin E supplementation leads to an increase in resistance to infectious diseases in humans remains to be demonstrated. Epidemiological studies that suggest a protective effect of vitamin E against cancer cannot be interpreted solely on the basis of possible effects on the IMMUNE SYSTEM, because protection against cell damage in general is also involved in the response to vitamin E. However, studies show that vitamin E can inhibit angiogenesis, a crucial requirement for tumour growth, *via* suppression of IL-8 and modulation of ADHESION MOLECULES [53].

Side effects

The evidence is compelling that intake of vitamin E above the recommended daily allowance (RDA) is of benefit to health. Vitamin E, given at recommended concentration orally, has a very low toxicity. The RDA in the USA is 10 mg or 15 IU. At daily doses up to 3000 mg vitamin E is without any significant side effects. However, there may be an upper limit for immunostimulation, since 300 mg/day vitamin E depressed bactericidal activity and proliferation of peripheral LEUKOCYTES in humans [54].

Phenolic compounds as immunomodulators

Many epidemiological studies have shown that a diet rich in fruits and vegetables can protect against the development of cardiovascular disease [55]. Researchers have examined the composition of these foods, and identified the physiologically active components as phytochemicals. Plant phytochemicals can be divided into plant sterols, FLAVONOIDS, and plant sulphur compounds. FLAVONOIDS are a

group of naturally occurring compounds that are widely distributed in nature and are ubiquitous in vegetables, berries and fruits and provide much of the flavour and colour to fruits and vegetables. At present, more than 500 FLAVONOIDS are known and described, while probably more than 4000 are present in various plants and extracts. A large number of studies have demonstrated the beneficial effects of flavonoid consumption against the development of cancer and cardiovascular disease. The general opinion, however, is that these compounds can serve to help prevent diseases, but are not effective enough to be used as specific therapies.

Flavonoids

History

The FLAVONOIDS were first isolated in the 1930s by Albert Szent-Gyorgyi, who also discovered vitamin C. Szent-Gyorgyi found that FLAVONOIDS strengthened capillary walls in ways in which vitamin C could not and, at first, they were referred to as vitamin P. But the chemical diversity of FLAVONOIDS precludes their classification as a single vitamin.

Chemical constituents

The FLAVONOIDS have been divided into six major groups: anthocyanidins, catechins or flavanols, flavones, flavonols, flavanones and isoflavones.

The biosynthesis of FLAVONOIDS in plant tissues has been extensively studied in many plants, and several biosynthetic steps have been elucidated. The general metabolism includes shikimic acid, l-phenylalanine and p-coumaric acid. The scheme is given in Figure 5. FLAVONOIDS include flavanones, flavones, chalcones, dihydroflavonols, flavonols, and any glycosides with an aglycone based on a C6-C3-C6 structure. Dihydroflavonols are included in the flavone and flavonol class. Here, we consider the well-investigated FLAVONOIDS from tea and single nutraceutical compounds that have been subject to the most extensive recent studies: coumarins, quercetin, rosmarinic acid and resveratrol.

Flavonoids from tea

History

Tea (*Camellia sinensis*) (Fig. 6) has been consumed as an infusion for millennia. The first documented use of this particular drink is dated 2700 BC, while the first report of its beneficial effects on human health was written in 1211 by the Japanese monk Eisai [56]. In the 16th century, European explorers used tea extracts to fight fever, headache, stomach ache, and joint pain. Today, tea is for the most part simply considered a tasty drink, but the scientific community has recently re-discovered the therapeutic potential of this beverage.

Tea is known as a rich source of antioxidant polyphenols (catechins, flavones, theaflavins, and thearubigins) and many confer a cardioprotective effect by decreasing LDL oxidative susceptibility, inhibiting LDL lipid peroxidation. Common green and black tea leaves consist of about 25–30% FLAVONOIDS, while the primary sources of polyphenols in green tea are catechins and flavones. Flavones include epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG) [57, 58].

Pharmacology

These compounds from tea have been tested *in vitro*, and EGCG especially shows inhibitory activity on metallo- and serine proteases that are involved in matrix degradation and act as crucial factors in tumour invasion. In animals, green tea significantly increases the activity of antioxidants and detoxifying enzymes, such as glutathione S-transferase, catalase, and quinone reductase, in the lungs, liver, and small intestine [56–58].

Under physiological conditions, EGCG and other selected polyphenols are converted to metabolites that result in remarkably increased inhibition of telomerase, an enzyme involved in cancer. In nude mouse models bearing both telomerase-dependent and -independent XENOGRAFT tumours cloned from a single human cancer progeny, only the telomerase-dependent tumours responded to prolonged oral administration of EGCG [59].

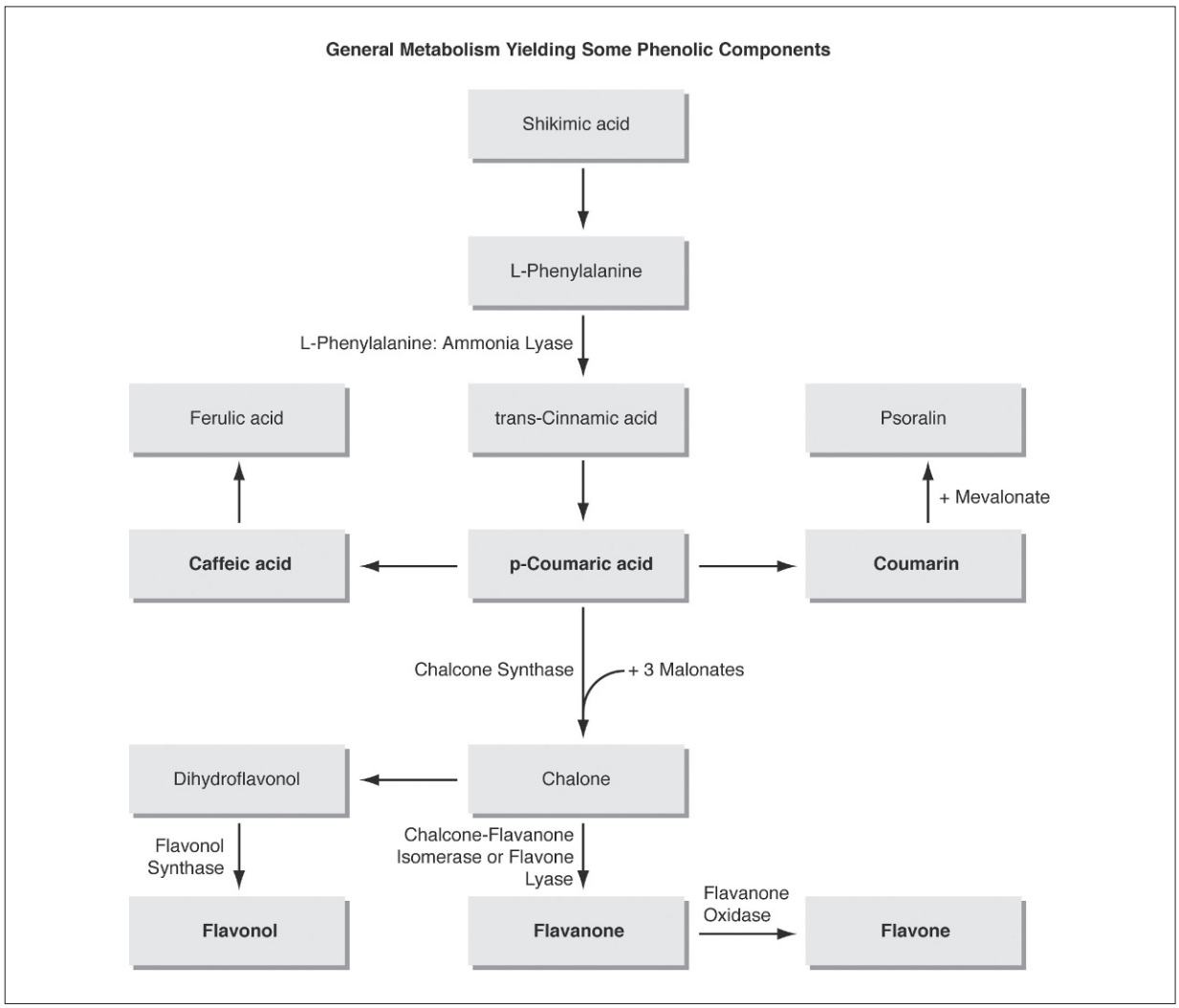

Figure 5. Metabolism of shikimic acid in plants to produce various flavonoids.

Immunomodulatory effects also have been observed with aqueous extracts of the tea, *Camellia sinensis*. Neopterin production (a sensitive marker of cell-mediated immunity) was slightly enhanced in unstimulated human peripheral mononuclear cells *in vitro*, whereas, on stimulation with mitogens, a reduction in neopterin formation was seen [60].

Several studies have shown that polyphenolic compounds isolated from green tea afford protection against UVB-induced inflammatory responses and photocarcinogenesis in murine models. Topical application of (–)-epigallocatechin-3-gallate (EGCG) before UVB exposure protected against UVB-induced immunosuppression and tolerance induction by: (i) blocking UVB-induced infiltration of $CD11b^+$ cells into the skin; (ii) reducing IL-10 production in skin as well as in draining lymph nodes (DLN); and (iii) markedly increasing IL-12 production in DLN [61]. These modulatory effects of EGCG could potentially protect against UVB-induced photocarcinogenesis. However,

Figure 6. Tea (*Camellia sinensis*)

clinical trials of green tea in cancer have not demonstrated clear beneficial effects. It is likely that the alleged antitumour actions require prolonged exposure to the agent. Inhibition of proteolytic enzymes to prevent metastases, alterations in cell communication, and anti-angiogenesis have all been touted as explanations for antineoplastic effects of green tea *in vitro* and in animals. However, many of these mechanisms lead to tumour regression only after prolonged exposure to an antineoplastic agent [62].

Black tea also contains smaller concentrations of quercetin and theaflavins that inhibit growth of virally transformed human lung cells and cell proliferation of a colon-cancer cell line [63]. This inhibitory effect appears to be the result of induction of APOPTOSIS and inhibition of the expression of the COX-2 gene.

Cellular pharmacokinetics

No studies on cellular pharmacokinetics of tea constituents have been performed, but EGCG is well absorbed into the plasma after repeated oral administration of EGCG or green tea extract to human volunteers [64].

Clinical indications

Green tea is an herbal medicine used in the adjuvant treatment of cancer, dental plaque, and heart disease. Green tea extracts are recommended for general use at 300–400 mg daily, which when "translated" into consumption of tea as a beverage, means about three cups daily. Recently, it was reported that the intake of catechin, present in green tea, significantly suppresses the expression in ischaemic heart tissue of inflammatory factors, including ADHESION MOLECULES, CYTOKINES and matrix METALLOPROTEINASES (MMPs) [65]. These are all known to be regulated by NF-κB, which is a central signalling molecule in inflammatory processes. Although catechins are not specific inhibitors of NF-κB, they have similar effects to selective inhibitors of ADHESION MOLECULES and other inflammatory agents. Therefore, catechins have the potential to suppress clinical inflammatory reactions.

Side effects

Taking green tea as capsules or beverages can provoke allergic reactions. Other more severe side effects include muscle spasms or twitches, nervousness, insomnia (sleeplessness), rapid heart rate, high levels of stomach acid, and heartburn (due to the caffeine content in the tea). Green tea may change the way iron is used in the body and there are some cases of anaemia in children drinking an average of 250 mL green tea per day.

Single nutraceutical compounds

Coumarins

Quite apart from their well-known therapeutic uses as anti-coagulants, plant-derived coumarins have received particular interest as potential sources of anti-inflammatory and immunomodulatory drugs. The coumarin scaffold (Fig. 7) has often served as a starting point for the development of new chemical entities by medicinal chemists. The whole class of compounds possesses a range of different pharmacological activities including anticancer, antioxidant, anti-inflammatory, antiviral, anticoagulant, antibacterial, analgesic and immunomodulatory effects [66]. They exert many of their effects by inhibiting cell proliferation, interfering with mitotic spindle

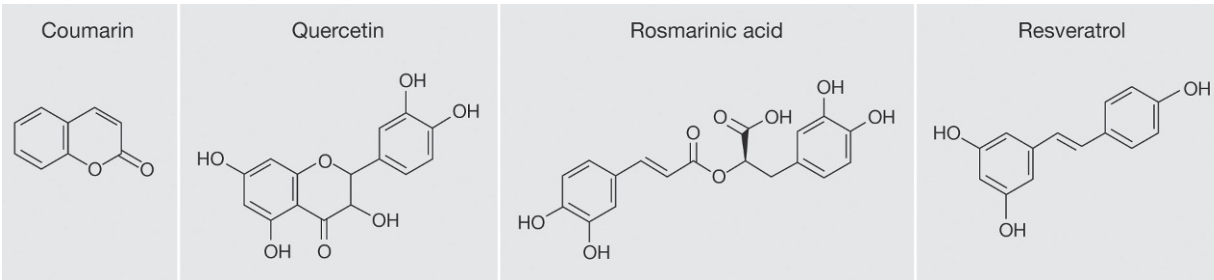

Figure 7. Structures of selected antioxidant nutraceutical agents

microtubule function, decreasing MMP activity, and blocking the cell cycle in the S or G2/M phases to interfere with processes of cell division [67]. They also suppress free oxygen radical generation in leukocytes, inhibit different protein kinases and induce carcinogen-detoxifying enzymes. Attenuation of the phosphorylation of Akt/PKB and inhibition of COX have been proposed as potential mechanisms of inhibition of inflammation [66]. Coumarins possessing antifungal and antibacterial activities have been described [68, 69]. The generally poor oral bioavailability of these compounds can be overcome by novel formulations (e.g. nanoparticles, cyclodextrin inclusions) or by linking the molecules to carriers designed for targeted delivery.

Side effects reported with this class of compounds include some non-haemorrhagic responses (i.e. with indanedione derivatives), as well as haemorrhagic events. Therefore, their clinical use should be carefully monitored.

Quercetin

Present in many foods (vegetables, fruit, tea, honey and wine), the flavonoid quercetin (Fig. 7) is one of the most prominent dietary antioxidants. It is claimed to exert various beneficial health effects. These include protection against cardiovascular diseases, sarcoidosis, certain forms of cancer (see Tab. 2), pulmonary disease and osteoporosis. In addition to being antioxidant, it also exerts anti-inflammatory, antiproliferative and gene expression regulating effects (Fig. 8). Quercetin modulates T helper cell function, inhibits lymphocyte activation and proliferation and induces apoptosis in leukaemia cells, an action to which healthy peripheral blood mononuclear cells are much less sensitive [77, 78]. It is thought that the majority of its beneficial effects are mediated by modulation of radical-induced cell membrane damage. However, most of these studies have been performed with immortalised or cultured cell lines *in vitro*. Only its antioxidative and anti-inflammatory effects have been confirmed *in vivo* [76].

Absorption of quercetin was thought to be restricted to passive diffusion of the aglycone (without a sugar substituent) across the gastrointestinal tract. It is now known that conjugation with a glycoside considerably enhances absorption [79]. Thus, after repeated supplementation, human quercetin plasma concentrations can attain the high nanomolar or low micromolar range. So far, toxicity of quercetin has only been reported *in vitro* (Fig. 8). The effects observed are thought to be due to toxic oxidation products, which are likely to be metabolised *in vivo* [76].

Rosmarinic acid

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Fig. 7). It is commonly found in species of the *Boraginaceae* and the subfamily *Nepetoideae* of the *Lamiaceae*, but also in other higher plant families and in some fern and hornwort species. Its phytobiosynthesis starts with the amino acids, l-phenylalanine and l-tyrosine [80]. Rosmarinic acid exerts antiviral, antibacterial, anti-inflammatory and antioxidant effects. Its anti-inflammatory properties are thought to be due

Table 2. Effect of quercetin on different cancer cell lines (modified from [70])

Cell line [ref]	Quercetin effects
HL-60 [71]	Inhibition of cytosolic and membrane kinases (PKC and TPK) Complete repression of phosphoinositide (PI, PIP, and PIP2) activity at sub-microgramme concentrations (10^{-7} M)
A-549 [72]	At low concentrations promotes cell proliferation and cytotoxic at higher concentrations At lower concentrations increases total antioxidant capacity (TAC) of cells, leading to a progressive decrease in TAC at higher concentrations
K562 [73]	Suppresses expression of oncogenes (c-myc and Ki-ras) Causes depletion of inositol-1,4,5-triphosphate (IPs)
Glioma cell [74]	Causes arrest at the G2 checkpoint of the cell cycle Decreases the mitotic index
MCF-7 [75]	Cytotoxic at μM concentrations Causes cell cycle arrest in G2/M phase Inhibits tumour growth *in vivo* (mice grafted with mammary carcinoma)

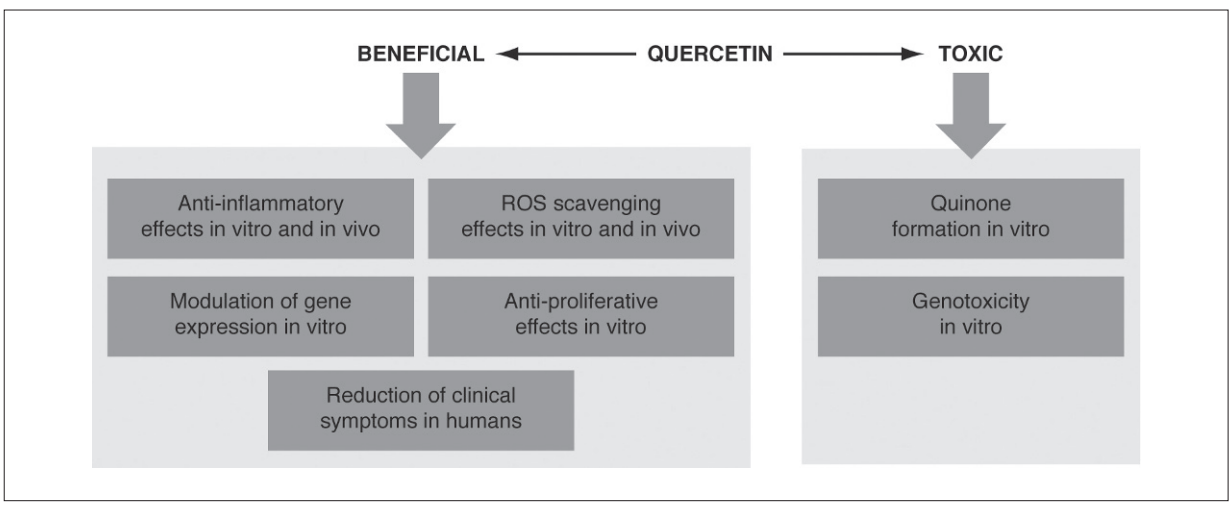

Figure 8. Quercetin – beneficial and toxic effects exerted *in vivo* and *in vitro*
(from [76] with permission from Elsevier)

to: (i) inhibition of lipoxygenases and COXs, (ii) interference with the complement cascade and (iii) inhibition of Lck, p56, a tyrosine kinase involved in T lymphocyte signalling [81,82]. Like other phenolic compounds, it may protect cells from undesired transformation.

Rosmarinic acid has poor oral bioavailability and is rapidly eliminated from the blood circulation after intravenous administration, showing very low toxicity [81]. Formulation to increase oral bioavailability would considerably enhance its therapeutic potential.

Resveratrol and red wine

While excessive alcohol consumption is well known to be harmful to health, mild-to-moderate red wine drinking has been linked with reduced cardiovascular, cerebrovascular, and peripheral vascular risk, giving rise to the popular concept of the French Paradox [83]. Moderate red wine consummation can also prevent undesired cell transformation that could potentially lead to cancer. A large number of reports in the literature indicate that resveratrol present in red wine is primarily responsible for the cardioprotection. Resveratrol (*trans*-3,4',5-trihydroxystilbene; Fig. 7) is a member of a family of polyphenols called viniferins. It is biosynthesised from one molecule of *p*-coumaroyl CoA and three molecules of malonyl CoA by stilbene synthase present in higher order plants, such as eucalyptus, spruce, lily, mulberries, and peanuts. Several recent studies indicate that resveratrol may provide protection against a wide variety of mechanisms, such as premature aging, impaired immune function, persistent chronic INFLAMMATION and cardiovascular dysfunction, involved in the development of degenerative diseases [84, 85]. This broad spectrum of potential EFFICACY is based mainly on the fact that resveratrol (like calorie restriction) indirectly activates SIRT1, the prototype of the sirtuins. These are an NAD(+)-dependent group of deacetylases that regulate longevity in lower organisms and, in mammals, control cellular stress resistance, genomic stability, tumourigenesis and energy metabolism. In yeast, resveratrol prolongs lifespan and in mammals it promotes glucose and lipid metabolism [85]. Resveratrol also modifies APOPTOSIS, inhibiting at very low concentrations and facilitating at higher doses.

Oral BIOAVAILABILITY of resveratrol is low. A new compound, SRT501, comprises a formulation of resveratrol with fivefold increased BIOAVAILABILITY. A clinical trial of SRT501 in type 2 diabetics resulted in a significant lowering of fasting plasma glucose and insulin; a trial in cancer patients is in progress [86].

Altogether, the FLAVONOIDS are an important reminder that the nutritional benefits of wholesome foods go beyond familiar vitamins and minerals. Although it may be convenient to reach for a high-potency flavonoid tablet for a particular disease, the best way to obtain a broad, healthy selection of FLAVONOIDS is by eating fresh fruit and vegetables, tea, honey, and soya.

Emerging therapies and summary

Throughout history people have used plants and nature-derived products to cure and prevent diseases. Although the healing properties of plants have been known for a long time, the ability to better exploit the uniqueness of plant therapeutics has been acquired only recently as a result of the dramatic developments in biochemical engineering, molecular genomics, analytical chemistry separation techniques, molecular characterisations and screening for new pharmaceuticals. The discovery, development and manufacturing of botanical therapeutics, either isolated from plants and different organisms or delivered as food constituents, is becoming a major area of expansion in plant biotechnology.

In addition to the search for the active immunostimu1atory agents in extracts of *Echinacea purpurea*, *Viscum album* and *Eleutherococcus senticosus*, a wide variety of plants are under investigation worldwide for immunostimu1ants, antibacterial and anticancer constituents. It is likely that with increasing emphasis on self medication to reduce health budgets, the commercial importance of plant and dietary immunostimulants for the therapy and continuous prophylaxis of mild infectious and immune disorders will increase.

Based upon the role of selenium in GPx, an anti-inflammatory benzisoselenazolone, ebselen (harmokisane), with GPx-like and PEROXYNITRITE scavenging activity, was developed clinically for cerebral ischemia, although it was not marketed. The discovery of this compound stimulated a search for other selenium-based anti-inflammatory or immunomodulatory agents [87]. Ebselen and a variety of other seleno-organic compounds have been found to be cytokine inducers *in vitro* and *in vivo* and have been proposed as potential antiviral agents [88].

The IMMUNE SYSTEM is subjected to a wide variety of stress factors in western society. These include overwork, lack of exercise, air pollution and processed

foods. Although it is often financially impracticable to perform extensive clinical studies on mild immunostimulants, it is widely agreed that in view of these stress factors, the benefit of dietary antioxidants in nutritional excess is probably greater than was previously thought. Extensive clinical studies on vitamin C and vitamin E suggest that there are indeed some prophylactic health benefits. Therapeutic prospects for the therapy of chronic degenerative or inflammatory diseases are also apparent from zinc supplementation. Even greater benefits are expected from the chemical modification of specific plant phenolic constituents such as quercetin. The poor oral BIOAVAILABILITY of many plant-derived compounds may be circumvented by suitable formulation and here SRT501 shows promise.

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www.mskcc.org/aboutherbs. Memorial Sloan-Kettering Cancer Center – About Herbs, Botanicals and Other Products

www.positivehealth.com/permit/Articles/Nutrition. Positive Health. Complementary Medicine Magazine

www.naturalstandard.com. Natural Standard. US database containing evidence-based information about complementary and alternative therapies

www.who.int/vmnis/en/. World Health Organisation - Vitamin and Mineral Nutrition Information System

www.eatright.org. American Dietetic Association

www.sirtrispharma.com. Sirtris, a GSK daughter company, developing small molecule drugs that target the sirtuins, a recently discovered family of seven enzymes associated with the aging process

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C10

Influence of antibacterial drugs on the immune system

Marie Thérèse Labro

Introduction

Interference of antibacterial agents with the IMMUNE SYSTEM, and its possible clinical implications, has long been a focus of attention worldwide. Toxic effects with immunological implications (NEUTROPENIA, ALLERGY, etc.) exerted an influence on the therapeutic choice early in the antimicrobial era, but attention has gradually shifted to beneficial "immunomodulatory" properties. Many papers in this field have been published and it has been periodically reviewed [1, 2]. Interest in "immunostimulation" peaked in the 1970s. Only recently have the potential benefits of down-regulating immunomodulators entered the limelight, with the understanding that immune hyperactivation (in sepsis and inflammatory/AUTOIMMUNE DISEASES, for example) can also have disastrous consequences. Incidental observations that some non-infectious diseases, including inflammatory disorders, may be improved by antibacterials have bolstered interest in the immunomodulatory activity of this class of drugs. An improved knowledge of the IMMUNE SYSTEM and its regulation, as well as technological advances, has facilitated such investigations. With a growing number of supportive experimental and clinical studies, the relevance of the immunomodulatory actions of some antibiotics for their therapeutic EFFICACY in various diseases is now generally admitted.

Antibacterial agents and the immune system

Two clinically relevant effects of antibacterial agents (ABA) are currently recognized: toxic or immunotoxic effects and intracellular BIOACTIVITY. Allergic reactions to antibiotics are commonly reported. Various reviews have addressed the literature on the diagnosis of drug HYPERSENSITIVITY reactions, which can be classified as immediate or non-immediate according to the time interval between the onset and the last drug administration [3, 4]. A Drug ALLERGY and HYPERSENSITIVITY Database has been established. It is already available online in many different languages and can be accessed using a personal login [5].

Intracellular BIOACTIVITY results from many factors, particularly cellular uptake [6–8]. Other possibilities for host-(microbe)-ABA interplay include modulation of bacterial virulence (leading to antibacterial synergy or proinflammatory effects); ABA activation/inactivation by host cell activities; and modulation of cell functions or products by ABA (resulting in immunostimulation, immunodepression or anti-inflammatory activity). These general aspects of ABA immunomodulatory effects are reviewed in [1, 2] and summarized here in Table 1 [1–8].

Modulation (stimulation/inhibition) of host cell activities is the most widely investigated and controversial aspect of ABA pharmacology. *In vitro* effects depend on the cell type, the technique, and many variables such as the drug concentration and cell activation status. These may differ from those occurring *ex vivo*/*in vivo*, which result from the combined activities of the different players: the host and its cellular and humoral (redundant) effectors, the pathogen and its virulence mechanisms, and the physicochemical, pharmacokinetic (concentration, tissue distribution, metabolism) and antibacterial properties of the ABA. *In vivo* studies are the gold standard, but are subject to multiple pitfalls such as species differences in the composition and functions of the IMMUNE SYSTEM [9], as well as ethical problems and inter-individual variability. Despite these difficulties, major progress has been made in develop-

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Table 1. Possible interactions of ABA with the immune system

Effects	Consequences	Main ABA
ABA effects on Host		
Toxic/immunotoxic side effects	Agranulocytosis, allergy auto-immunity	β-Lactams, chloramphenicol sulfonamides, minocycline, rifamycin, isoniazid
Cellular uptake	Intracellular bioactivity,* targeted delivery	Macrolides, quinolones, ansamycins
Alterations of immune cell functions	Altered cell activity (↑ bacterial killing, ↓ inflammation)	All classes
Scavenging/inhibition oxidants/ enzymes	Cell/tissue protection	β-Lactams, cyclines, isoniazid, clofazimine, rifampicin
Alteration of non-immune cell functions, gene expression	Altered immune system activity	Macrolides? Cyclines? Ansamycins?
Disturbances of host microflora	Superinfections toxin-mediated diseases Defective immune response?	
ABA effects on pathogens		
AB effect (cidal/static)	Decreased bacterial load (↓ specific response?)	All ABA bactericidal ABA?
Morphology**	PMN apoptosis/necrosis/stimulation	some β-Lactams
Release of proinflammatory mediators	cell stimulation (shock)	β-Lactams, Quinolones? Aminoglycosides
Decreased production of virulence factors	Increased susceptibility (PALE)	All ABA (sub/supra MICs)
Alteration of Ag structure	Altered specific response (relapses, chronic inflammation)	?
Binding to LPS	Decreased inflammation	Polymyxin B
Host effects on ABA		
Alteration of ABA (Metabolism, oxidation..)	Altered AB activity, toxicity	Chloramphenicol, isoniazid
Host effects on pathogens		
Intracellular sequestration	Decreased AB activity	Non-cell-penetrating AB
Alteration of structure or metabolism	Altered AB activity, synergy	Macrolides, quinolones, β-lactams

**Intracellular bioactivity results from many factors, including cellular uptake*
***Filament, spheroplast*
AB, antibacterial

ing immunomodulatory ABA, as described below (see section *Non-antibacterial effects of antibacterial agents: Therapeutic implications*). A simplified representation of the immunomodulatory profile of ABA is given in Figure 1 (modified from [1]).

Class-specific immunomodulatory effects

A very early study suggested that the immunomodulatory properties of antimicrobial drugs could be predicted from their mode of action on microbial cells [10]. However, this hypothesis has not been confirmed. Within a given class of drugs, variable effects are obtained, depending on the particular chemical structures and physicochemical properties. This chapter describes the main effects of ABA (presented in alphabetical order) on immune functions.

The chemical structure of lead compounds in the most investigated ABA classes (e.g. ansamycins, β-lactams, cyclines, macrolides and quinolones) as well those of chemically modified tetracyclines (CMT-3, tigecycline) and of a chemically modified macrolide (EM703) are given in Figure 2. A synthesis of *in vitro*, *ex vivo* and *in vivo* effects is given in Tables 2–4 for three ABA families that have demonstrated class-dependent effects (e.g., ansamycins, Tab. 2; cyclines, Tab, 3; and erythromycin A-derived macrolides, Tab. 4).

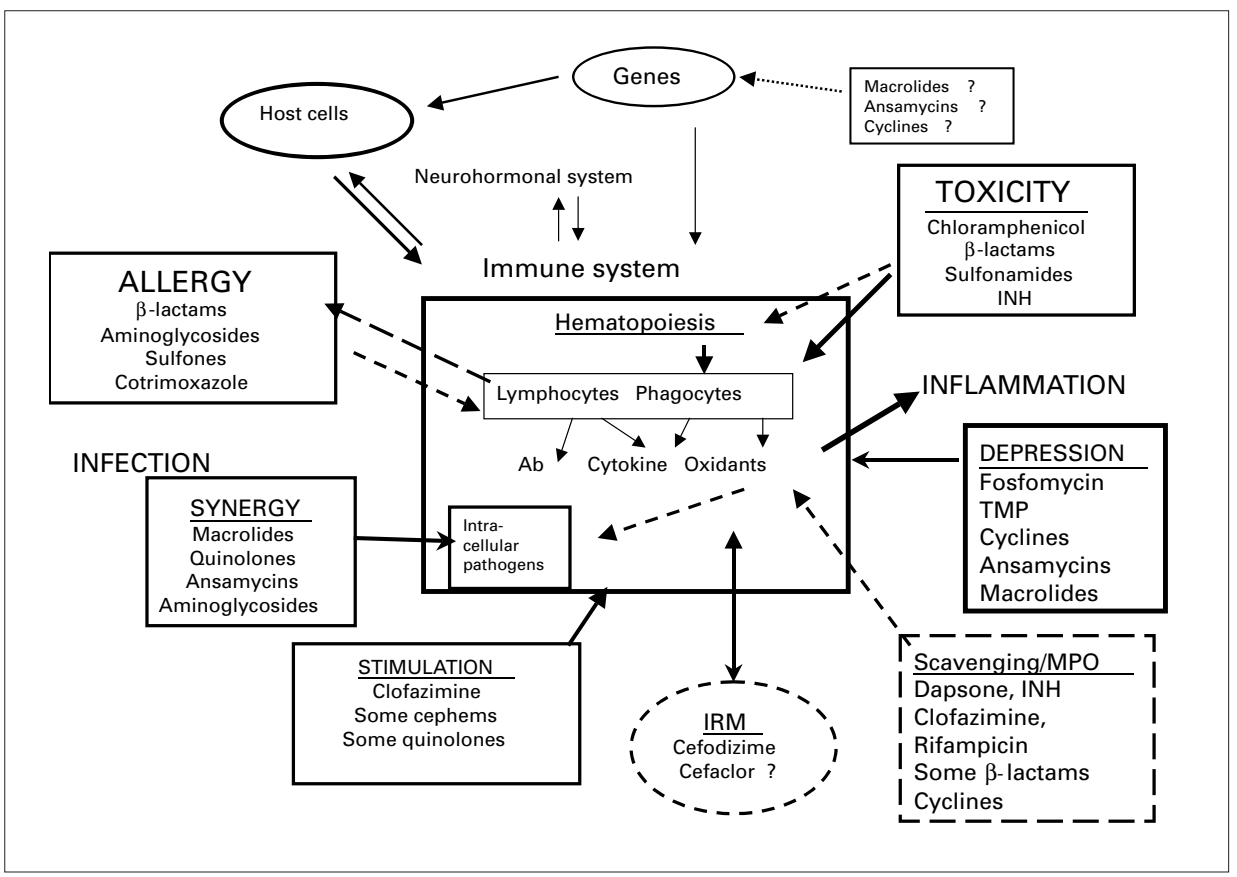
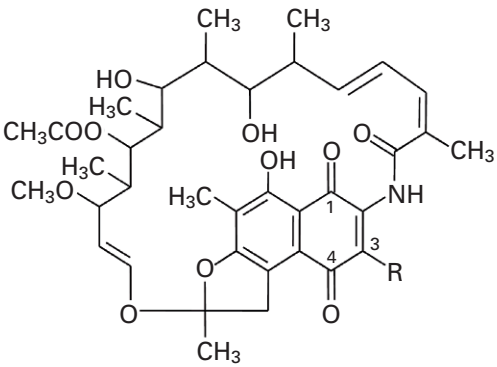
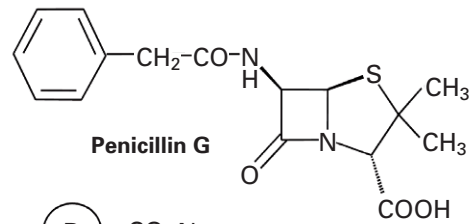


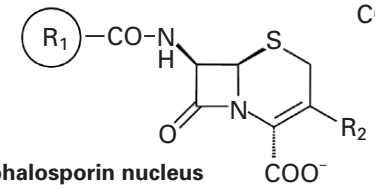
Figure 1. Schematic classification of antibacterial agents as immunomodulators



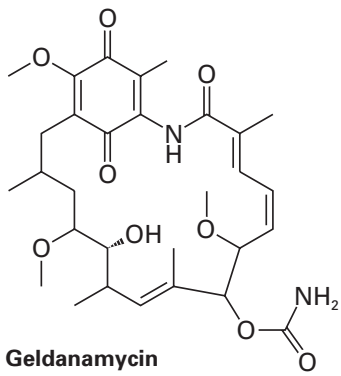
Ansamycin nucleus



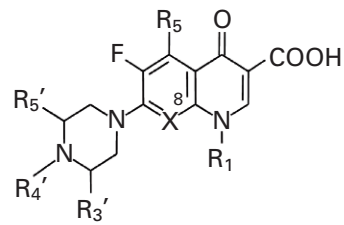
Penicillin G



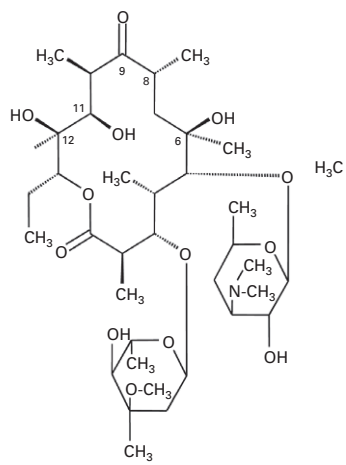
Cephalosporin nucleus



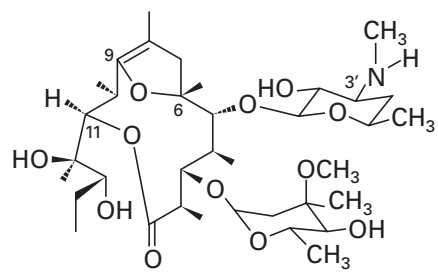
Geldanamycin



Quinolone nucleus



Erythromycin A



EM 703

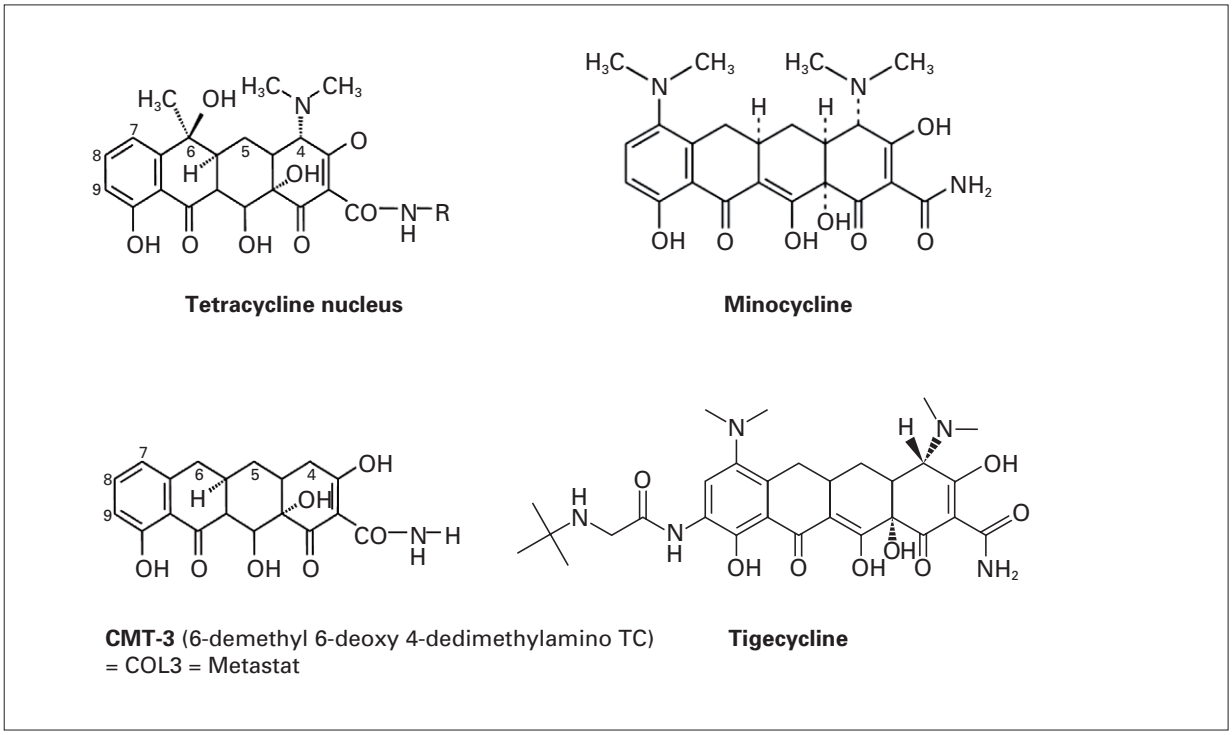


Figure 2. Chemical structures of important lead compounds
Ansamycin nucleus (and geldanamycin), cephalosporin nucleus, penicillin G, tetracycline nucleus (and minocycline, CMT-3 and tigecycline), quinolone nucleus (7-piperazinyl derivatives), erythromycin A (and EM703).

Aminoglycosides

Aminoglycosides interfere with bacterial protein synthesis by acting on the 30S ribosomal subunit. Although they are considered to be extracellular antibacterial agents, they accumulate slowly in host cells (over days in MACROPHAGES) by fluid-phase pinocytosis. The use of gentamicin as an extracellular bactericidal agent in many cell systems has been questioned [11]. Streptomycin BIOACTIVITY on intracellular *Escherichia coli* is thought to rely on stimulation of (and possible synergy with) macrophage oxygen-dependent bactericidal mechanisms. Aminoglycosides are still the "gold standard" for several intracellular infections [7].

Conflicting data have been published on the *in vitro* inhibitory effect of aminoglycosides (at therapeutic concentrations) on polymorphonuclear neutrophil (PMN) CHEMOTAXIS, oxidative metabolism and yeast killing. The underlying mechanisms include binding to negatively charged membrane phospholipids (leading to membrane disturbances); specific binding to inositol biphosphate (resulting in phospholipase C inhibition); and protein kinase C (PKC) inhibition. Interestingly, amikacin (contrary to other aminoglycosides) enhances the PMN oxidative burst at low concentrations *in vitro*, whereas concentrations higher than 1 g/L inhibit this phenomenon, probably as a result of oxidant scavenging [12]. Gentamicin, but not amikacin, significantly decreases lymphokine-activated killer cell activity *in vitro*, at clinically relevant concentrations. However, no clinical evidence of these observations has been documented.

Cystic fibrosis (CF), a systemic autosomal recessive inherited disorder, results from mutations in the

Table 2. Effect of ansamycins on the immune system

In vitro	*Ex vivo/in vivo*
Cellular uptake: 2–14-fold accumulation (rifampicin) 61–88-fold accumulation (rifapentine)	Antibacterial activity (*Mycobacteria*, intracellular pathogens)
Phagocyte functions: Decreased oxidants, chemotaxis Decreased leukocyte-induced angiogenesis Decreased NO	Anti-inflammatory? Decreased oxidants? (meningitis model)
Scavenging of $O_2^{-\cdot}$	
Immunodepression: Reduced PHA/ConA-induced proliferation Reduced IFN-γ production Increased CD1b expression (macrophage membrane)	Decreased cellular immunity Increased Ab against SRBC Increased mycobacterial Ag presentation?
Other effects: Reduced iNOS mRNA (geldanamycin) Decreased collagenase activity (rifamycins) Decreased cholesterol	Suppressed disease onset (EAE model) (geldanamycin), adjuvant- induced arthritis, anti-inflammatory? (geldanamycin), atherosclerosis model (CGP43371) Increased P-glycoprotein expression (human gut) Enhanced MDR1 mRNA (lymphocytes) (resistance of tumor cells?)
Possible mechanisms	
Antibacterial activity	
Immunomodulatory effect ligand for fMLP-R (PMN) superoxide anion scavenging decreased NF-κB activation (rifamycin SV) activation of glucocorticoid receptor	
Geldanamycin (and derivatives) Decreased tyrosine kinase activity, induces heat shock reaction, raised Iκ-B mRNA.	

cystic fibrosis transmembrane conductance regulator (CFTR) gene. In class I defects the CFTR mutation results in a premature stop codon which interferes with translation of CFTR. Distinct aminoglycosides (specifically gentamicin but not tobramycin) have been shown to bind to the rRNA, causing misreading of the abnormal stop codon and insertion of alternate amino acids that then restore full-length translation. In cell lines, gentamicin treatment restores full-length CFTR to 10–20% of normal levels. Several trials have given encouraging results after intravenous administration of gentamicin [13], but intranasal administration was less successful [14]. Research is continuing on the regulation of premature termina-

Table 3. Effect of cyclines on the immune system

In vitro	*Ex vivo/in vivo*
Cellular uptake: 2–4-fold accumulation Na-dependent transport system 60-fold concentration (minocycline)	
Phagocyte functions: Decreased oxidants, phagocytosis, CT Increased IL-1β (LPS-stimulated monocytes) (minocycline > tetracycline)	Decreased oxidants (myocardial infarction, acne, inflammation) Reduced inflammatory damage (*H. pylori*-associated gastritis) Decreased TNF-α, IL-1β (LPS challenge) Prevention of endotoxic shock/tetracycline)
Non-immune cells: Decreased NO (minocycline only) (chondrocytes) Reduced PAR2-IL8 axis (keratinocytes) Decreased proinflammatory cytokines (microglia) Reduced angiogenesis (minocycline, doxycycline) Increased proliferation of mouse thymic epithelial cells	Reduced iNOS, caspase 1 (minocycline: mouse model of Parkinson's disease) Inhibited animal models (ischemia/reperfusion injury, stroke, chronic colitis, EAE, Japanese encephalitis, Huntington's disease, human amyotrophic lateral sclerosis, congenital muscular dystrophy type 1A) (minocycline)
Decreased IgE production (PMBC from asthmatic patients)	
Scavenging of HOCl	
Reduced MMP activity Reduced MMP-9 (protein, mRNA) (THP-1 monocytes, doxycycline) Reduced gelatinase, collagenase (PMN, tumor cells) Decreased T leukemia cell growth (apoptosis/↓MMP-2 and caspase-3 activation)	Inhibited animal models of multiple sclerosis, DSS- and TNBS-induced acute and chronic colitis Reduced MMP-9 (protein, mRNA) in patients with AAA treated with doxycycline)
MDR1 overexpression (breast carcinoma cells)	Increased resistance of cancer cells?
?	Minocycline-induced autoimmune syndromes Hypersensitivity reactions
Mechanisms?	
Antibacterial activity *(C. pneumoniae, Mycoplasma, Propionibacterium,* others?*)*	
Immunomodulatory effect Ca^{2+} chelation, Mg^{2+} binding Photodamage Scavenging HOCl PKC inhibition Ras-ERK/mitogen-activated protein kinase (MAPK) signaling pathway	

Table 4. Effect of erythromycin A-derived macrolides on the immune system

In vitro	*Ex vivo/in vivo*
Cellular uptake: >10–>200–300-fold accumulation (active mechanism, related to P-gP?)	Tissue accumulation, intracellular bioactivity
Phagocyte functions: Decreased oxidants, CT? ↑ degranulation Cytokines: reduced TNF-α, IL-1β, IL-8, IL-6; increased IL-10 decreased LTB4 and NO, increased apoptosis enhanced antigen-presenting cell activity, macrophage polarization shifted towards the alternatively activated phenotype	Anti-inflammatory? Short-term treatment: increased phagocyte responses Long-term treatment: decreased phagocyte responses Various animal models: decreased PMN migration, adhesion molecules and inflammatory cytokines. IL-10-deficient mice: reduced development of severe colitis and of production of IFN-γ and IL-12 Patients: DPB, asthma, sinusitis, CAD (decreased inflammatory parameters: cytokines, PMN infiltration, adhesion molecules, NO levels, etc.)
Reduced angiogenesis	Reduced tumor growth
Decreased glycoconjugate secretion (human airway cells)	Reduced bronchial responsiveness Decreased mucus production
Reduced proliferation (fibroblasts)	
Cancer cells: reduced proliferation? decreased production of TGF-β, TNF-α	Animal models: reduced metastasis and tumor growth, increased survival Lung cancer (humans): increased survival
Reduced metalloprotease activity	Cardiac allograft recipients: suppression of MMP-9
Reversed MDR	Increased P-glycoprotein (protein and mRNA)
Mechanisms?	
Antibacterial activity *(C. pneumoniae, Mycoplasma, H. pylori, P. aeruginosa, ?)* Bacterial virulence *Steroid-sparing effect*	
Immunomodulatory effect modulation of the PLD-PPH pathway (PMN); decreased Ca^{2+} influx inhibition of MAP kinase (e.g.ERK1/2) activity modulation of genes: induction of protein expression (P-gP, others), reduced NF-κB (T cells), AP-1 (bronchial cells)	
Altered gene expression	

tion codons and may open future directions for this innovative treatment strategy [15]. However, owing to the toxicity of aminosides, long-term treatment is not suitable for CF patients.

Ansamycins

Antibacterial ansamycins are a group of macrocyclic antibiotics containing a chromophoric naphthohydroquinone system spanned by a long aliphatic bridge (Fig. 2). They are mainly effective against mycobacteria, in which they alter RNA biosynthesis by interfering with RNA polymerase activity. Ansamycin-induced lupus syndromes have been identified, which in the case of rifampicin-induced lupus erythematosus is associated with COMBINATION THERAPY with clarithromycin or ciprofloxacin. All these drugs are metabolized through the cytochrome P450 liver enzyme system and their combined administration may lead to higher rifampicin blood levels.

In vitro effects

Cellular uptake of rifampicin, the most important representative of this group, is only moderate, but rifapentine, a cyclopentyl rifamycin derivative, is avidly concentrated (61–88-fold) by PHAGOCYTES, possibly through membrane binding. Ansamycins are active against intracellular pathogens in both normal and deficient PHAGOCYTES. They exert depressive effects on phagocyte and lymphocyte activities. They impair various PMN functions such as CHEMOTAXIS, through competition with PMN RECEPTORS for small chemoattractant peptides, as well as the oxidative burst (although light-absorbing activity and SUPEROXIDE ANION scavenging can interfere with the detection method) [16]. The most active compounds are derivatives carrying an acidic substituent at C3. PMN from patients with RHEUMATOID ARTHRITIS (RA) are more susceptible to the depressive effect of rifamycin SV than are cells from healthy subjects. Ansamycins may also impair specific immune responses such as phytohemagglutinin (PHA)- and concanavalin (ConA)-induced proliferation of peripheral blood cells and production of IFN-γ. Rifamycin B, rifapentine, rifamycin SV, rifabutin and rifampicin inhibit both TNF-α and PMA-induced NF-κB activation in Jurkat T cells [17]. The production of NITRIC OXIDE (NO) by the inflammatory isoform of NO synthase (NOS) in brain glial cells is thought to contribute to the development of neurological diseases and trauma. Activation of a heat SHOCK response (HSR) reduces NOS expression *in vitro*, and *in vivo* attenuates the clinical and histological symptoms of the demyelinating disease experimental autoimmune encephalomyelitis (EAE). Geldanamycin, a benzoquinoid ansamycin is a fungus-derived antibiotic with multiple pharmacological properties. It induces an HSR in rat astrocytes and glioma cells *in vitro* and concentration-dependently reduces NOS mRNA levels. In addition it inhibits tyrosine kinase activity and increases mRNA levels of the inhibitory protein IκBβ, suggesting that inhibition of NF-κB activation underlies its IMMUNOSUPPRESSIVE EFFECTS [18]. The immunosuppressive potential of rifampicin may also be due to binding and activation of glucocorticoid RECEPTORS [19, 20].

Non-classical antigen presentation by CD1 molecules expressed on cytokine-activated MONOCYTES, and cell-mediated responses supported by double-negative (DN) $CD8^+$ responder αβT cells, are involved in host resistance against mycobacterial infection. Clinically achievable concentrations of rifampicin increase CD1b expression on the membrane, resulting from increased CD1b GENE EXPRESSION, without reducing DN T cell-mediated cytolysis of lymphoblastoid cells transfected with CD1b cDNA and pulsed with *Mycobacterium tuberculosis* [21, 22]. Rifampicin could thus improve mycobacterial antigen presentation, without impairing responder T cell function. Among other potentially immunomodulatory properties of ansamycins, inhibition of collagenase type XI has been reported.

In vivo/ex vivo effects

In a rabbit model of *Streptococcus pneumoniae* meningitis, NEUTROPHILS and MONOCYTES from rifampicin-treated rabbits produced smaller amounts of REACTIVE OXYGEN SPECIES than LEUKOCYTES from ceftriaxone-treated animals [23], possibly due to direct inhibitory effects on LEUKOCYTES present in cerebrospinal fluid. In accordance with *in vitro* data (see above), a single

injection of geldanamycin prevents disease onset by over 50% in mice actively immunized to develop EAE [18]. This drug also confers protection against INFLAMMATION-associated acute lung injury, and suppresses the progression of adjuvant-induced arthritis.

Ansamycins are hypolipidemic compounds, and markedly lower high-density lipoprotein (HDL) cholesterol levels. CGP 43371, a new rifamycin derivative, has been tested for its hypolipidemic and anti-atherosclerotic activity in cholesterol-fed rabbits [24]. Compared with control rabbits, CGP 43371 treatment lowered total cholesterol and lipoprotein cholesterol levels and inhibited aortic atherosclerosis. CGP 43371-treated rabbits developed striking splenomegaly, with massive accumulation of macrophagic foam cells in the splenic red pulp. The authors speculated that CGP 43371 inhibits the development of atherosclerotic lesions in rabbits by both a hypolipidemic mechanism and by accumulation of macrophage foam cells in the spleen. The human MDR1 gene encodes for P-glycoprotein (P-gp), an ATP-dependent transmembrane protein that transports numerous neutral and cationic compounds out of many cells. Consequently, induction of P-gp is at least partly responsible for the development of resistance to chemotherapeutic regimens, and is involved in changes in both drug distribution and CLEARANCE. Despite inter-individual variability, a significant increase in the expression of multidrug resistance-1 (MDR1) mRNA in human LYMPHOCYTES has been observed following rifampicin administration. P-gp is also induced by rifampicin in the human gut, as shown by intestinal biopsy. In mice inoculated with Ehrlich ascites carcinoma cells, a treatment with rifampicin confers resistance to the antineoplastic drug doxorubicin, probably through an increased expression of P-gp [25].

The therapeutic potential of various ansamycins as anti-inflammatory agents has received some attention (see section *Therapeutic implications*).

Benzylpyrimidines: Trimethoprim and analogs

Benzylpyrimidines [trimethoprim (TMP), tetroxoprim, epiroprim and brodimoprim] inhibit dihydrofolate reductase to exert their antibacterial effects. TMP is generally used in combination with another antifolate, sulfamethoxazole (SMX). TMP accumulates 6–20-fold in host cells.

In most studies, TMP, alone or in combination, has an inhibitory effect on PMN functions. However, TMP-SMX increases NO production by PMN from patients with chronic granulomatous disease [26]. Brodimoprim, in which the methoxy group in position 4 of the TMP benzyl ring is replaced by a bromine atom, displays greater lipophilicity and cellular uptake (74-fold) than TMP, and no inhibitory effects on PMN functions.

β-Lactams

β-Lactam antibiotics comprise five groups of compounds: penams (penicillins, Fig. 2- and β-lactamase inhibitors), penems (faropenem), carbapenems (imipenem, meropenem), cephems (cephalosporins, Fig. 2, cephamycins, oxa-and carbacephems) and monobactams (aztreonam); all of which inhibit various enzymes (penicillin-binding protein) involved in PEPTIDOGLYCAN synthesis.

Understanding of the immunological mechanisms involved in non-immediate HYPERSENSITIVITY reactions to β-lactams has improved over the last few years, with better definition of the different T cell subpopulations involved. In delayed reactions to amoxicillin, DENDRITIC CELLS play a relevant role in inducing the T cell responses [27]. Several studies reported in Europe, China, and the United States on the association of β-lactam ALLERGY with genetic predictors converge in showing that immediate allergic reactions to β-lactams are influenced by genes of the IL-13 and IL-4 pathways, which are involved in IgE CLASS SWITCHING and atopy. β-Lactam antibiotics, predominantly amoxicillin, are also associated with acute generalized exanthematous pustulosis as an adverse (delayed cellular HYPERSENSITIVITY) effect.

In vitro effects

Cellular uptake of β-lactams is poor, but intracellular activity of the drugs has been observed in some models. Passive diffusion seems to be the predominant uptake mechanism with most penicillin deriva-

tives, at least in their ionized forms (weak organic acids). A proton-dependent dipeptide transport CARRIER has been described for some cephalosporins, and a probenecid/gemfibrozil-inhibitable organic anion transporter may be involved in penicillin G efflux from cells.

PMN may engulf staphylococci without effectively killing them. It has been proposed that *S. aureus* that have lived and survived in the intra-PMN environment may possess modified TARGET proteins for β-lactam antimicrobials, resulting in antimicrobial resistance. For instance, alterations of cefazolin targets (e.g. penicillin-binding proteins) by PMN have been forwarded to explain the decrease in the antibacterial activity of this cephalosporin *in vitro* and *in vivo* [28]. In contrast, bactericidal synergy between β-lactams and the specific and non-specific arms of the IMMUNE SYSTEM have been reported [29]. An indirect impact of β-lactams on PMN death after *E. coli* killing has been observed. β-Lactams that induce filament formation promote PMN necrosis, while imipenem, which induces spheroplast formation, promotes PMN APOPTOSIS [30].

Many data are available on the direct *in vitro* effects of these drugs on phagocyte functions, but no general class or subgroup effect has been demonstrated. In contrast, selective actions have been linked to certain chemical features. In addition, few and often controversial data have been reported on the effect of β-lactams on cytokine release. In general, all these effects do not support clinical relevance.

The amoxicillin-clavulanic acid combination, which increases the phagocytic and microbicidal activity of PMN, has been shown to enhance the production of IL-1β and IL-8 by LPS- and *Klebsiella*-stimulated PMN. Benzylpenicillin and serum from patients with sickle cell disease have a combined suppressive effect on normal lymphocyte production of IFN-γ and TNF-α, while increasing that of IL-2 [31]. Benzylpenicillin conjugates to IFN-γ and reduces its activity. It also conjugates to IL-1β, IL-2, IL-5, IL-13 and TNF-α, but without altering their biological activity [32].

In this context, special mention must be made of the 2-amino-5-thiazolyl cephalosporin, cefodizime, which was the subject of worldwide interest in the 1990s and was referred to as an IMMUNE RESPONSE MODIFIER antibiotic. Cefodizime stimulates the proliferative response of LYMPHOCYTES, increases the phagocytic and bactericidal activity of PMN, and down-regulates the production of proinflammatory CYTOKINES by stimulated MONOCYTES. Contrary to all other β-lactams, cefodizime significantly increases colony formation by granulocyte-monocyte progenitors. The chemical structure responsible for these immunomodulatory properties has been identified as the thio-thiazolyl moiety at position 3 of the cephem ring, but the cellular mechanism remains to be elucidated.

In vivo/ex vivo effects

Modulation of cytokine production by β-lactams has been observed *ex vivo*. IL-6 concentrations are significantly elevated after ceftazidime administration to both septic (independently of LPS levels) and non-septic rats, and TNF-α levels are also increased in non-septic rats, suggesting a direct effect of ceftazidime on cytokine production. However, in a randomized trial comparing imipenem and ceftazidime in the treatment of urosepsis, no differences in plasma ENDOTOXIN, IL-6 or TNF-α levels, or urinary ENDOTOXIN, IL-6 or IL-8 levels, were found between the two treatment groups [33], questioning the clinical relevance of this effect.

The immunomodulatory activity of cefodizime has been investigated *ex vivo* and *in vivo*, in both humans and animals (healthy and immunocompromised) [34]. Animal studies have demonstrated the benefit of cefodizime in the prevention and/or treatment of various infections caused by resistant pathogens, such as *Plasmodium berghei*, *Candida albicans* and *Toxoplasma gondii*, and its better EFFICACY compared to other cephalosporins in immunocompromised animals. *Ex vivo* studies have shown strain- and cefodizime concentration-dependent immune responsiveness with regard to inhibition of delayed-type HYPERSENSITIVITY, ANTIBODY production and lymphocyte proliferation. In healthy individuals given cefodizime, immune parameters are little affected, if at all, whereas in immunocompromised individuals (cancer, hemodialysis, old age, surgical stress, etc.) cefodizime administration restores depressed phagocytic functions.

Chloramphenicol

Chloramphenicol impairs bacterial protein biosynthesis by acting on the 50S ribosomal subunit. Owing to its lipid solubility, chloramphenicol accumulates moderately (2–9-fold) in host cells and is active intracellularly.

Chloramphenicol is a leading cause of NEUTROPENIA by CYTOTOXIC mechanisms involving its nitroso- and dehydro- derivatives. This limits the therapeutic application of the antibacterial. Reports regarding a potential chloramphenicol-induced reduction in host cell functions are conflicting. Chloramphenicol decreases lymphocyte functions *in vitro* and impairs ANTIBODY production *in vivo*. Thiamphenicol synergizes with PMN to increase their bactericidal activity.

Cyclines

Cyclines (Fig. 2) interfere with bacterial protein synthesis, by acting on the 30S ribosomal subunit. The tetracycline molecule has been chemically modified in multiple ways, generating a new family of compounds called CMTs (chemically modified tetracyclines) that lack antimicrobial but still retain anti-collagenase activity. The first of these CMTs, 4-de-di-methylaminotetracycline, circumvents the emergence of tetracycline-resistant microorganisms in the oral flora and gut after administration to experimental animals [35].

In vitro effects

Classically, cyclines are passively accumulated by PHAGOCYTES and exert intracellular activity. Recently, it has been shown that NEUTROPHILS express a saturable, sodium-dependent transport system that allows them to take up and accumulate MINOCYCLINE and other tetracyclines [36]. The observed cellular/extracellular concentration (C/E) ratios are greater than 60 for MINOCYCLINE and >7 for doxycycline.

Many reports confirm the inhibitory action of cyclines on various phagocyte functions at therapeutic concentrations. The underlying mechanisms include Ca^{2+} chelation, binding of intracellular Mg^{2+}, photodamage of PMN, and scavenging of hypochlorous acid. Structure-activity studies indicate a parallel increase in lipid solubility and inhibitory properties, although others stress the different chemical reactivity of the various molecules to UV light. Few studies have investigated the effect of cyclines on cytokine production. MINOCYCLINE and to a lesser extent, tetracycline, increased IL-1β secretion by LPS-stimulated human MONOCYTES. In contrast, IgE production by peripheral mononuclear blood cells (PMBC) from asthmatic patients was reduced when the cells were co-cultured with IL-4 and CD40 in the presence of MINOCYCLINE or doxycycline [37].

Other reports have described the interaction of cyclines with non-immune cells. MINOCYCLINE and doxycycline inhibit angiogenesis *in vitro* by a metalloproteinase-independent mechanism, with implications for the treatment of disorders linked to extracellular matrix degradation, including periodontal disease, arthritis and tumor angiogenesis. MINOCYCLINE (but not doxycycline or tetracycline) concentration-dependently decreases NO production in IL-1-stimulated chondrocytes, by inhibiting mRNA and protein expression of inducible NOS (iNOS) [38]. Tetracyclines also attenuate the PAR2 (protease-activated RECEPTOR 2)-IL-8 axis in keratinocytes and thereby effectively modulate proinflammatory responses in the skin [39]. Doxycycline enhances the proliferation of mouse thymic epithelial cells, which support positive thymocyte selection and are essential throughout the development of thymocytes. The increased cell proliferation is due to a shortened G_0/G1 phase. The underlying mechanism supports an active role of the Ras-ERK/MITOGEN-activated protein kinase (MAPK) signaling pathway as a TARGET for the cyclines [40]. Microglia are involved in Alzheimer's disease pathogenesis by promoting amyloid β plaque (Aβ) formation and production of proinflammatory CYTOKINES, but PHAGOCYTOSIS of Aβ by activated microglia may prevent Aβ-mediated neurotoxicity. MINOCYCLINE down-regulates the production of proinflammatory CYTOKINES by human microglia without affecting their beneficial PHAGOCYTOSIS of amyloid β fibrils [41].

An important property of cyclines is their inhibitory effect on various proteases. Doxycycline and CMTs inhibit the growth of a T lymphoblastic leu-

kemia cell line, by inducing APOPTOSIS *via* caspase-3 activation and matrix metalloproteinase (MMP)-2 inhibition [42, 43]. Cyclines impair collagenase/gelatinase activity, an effect that appears to be specific for neutrophil and tumor cell-derived enzymes. Doxycycline inhibits aortic smooth muscle cell MMP-2 production in part by reducing MMP-2 mRNA stability. As MMP activity contributes to extracellular matrix degradation in atherosclerotic plaque and abdominal aortic aneurysms (AAA), doxycycline has been proposed as a potential therapeutic agent for these diseases.

A possible molecular pathway of MINOCYCLINE concerns its inhibitory effects on PKCα/βII and transcription factors that regulate the expression of critical inflammatory genes such as MHC class II. Such a fundamental mechanism may underlie the pleiotropic effects of MINOCYCLINE in inflammatory disorders [44].

MCF-7 breast carcinoma cells treated with doxycycline overexpress P-gP and MDR1 mRNA, and accumulate the antineoplastic drug doxorubicin to a lesser extent than control cells. The use of doxycycline in cancer patients could induce P-gP expression by cancer cells, resulting in multidrug resistance.

In vivo/ex vivo effects

The therapeutic anti-inflammatory potential of cyclines is widely acknowledged (see section *Therapeutic implications*). Decreased oxidant production by PMN from patients with acute myocardial infarction and patients with acne treated with doxycycline or MINOCYCLINE has been observed *ex vivo*. Because cyclines inhibit several phagocyte functions, they have been assessed in animal models of ischemia-reperfusion injury and endotoxic SHOCK.

Much work has been done on the potential benefit of cyclines in various neurodegenerative and inflammatory disorders of the CENTRAL NERVOUS SYSTEM (CNS). MINOCYCLINE displays neurorestorative or neuroprotective properties in various models of neurodegenerative diseases. In particular, it has been shown to delay motor alterations, INFLAMMATION and APOPTOSIS in models of Huntington's disease, amyotrophic lateral sclerosis (ALS) and Parkinson's disease. MINOCYCLINE prevents nigrostriatal dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease [45]. The neuroprotective effect of MINOCYCLINE is associated with marked reductions in iNOS and caspase-1 expression in midbrain homogeneates of mice. MINOCYCLINE has been tested in mice expressing a mutant superoxide dismutase, linked to human ALS [46]. Administration of MINOCYCLINE to the diet, beginning at the late presymptomatic stage, delays the onset of motor neuron degeneration, muscle strength decline, and increases the longevity of mice by approximately 5 weeks for approximately 70% of tested mice. Moreover, less activation of microglia is detected at an early symptomatic stage and at the end stage of the disease in the spinal cord of mice treated with MINOCYCLINE. The benefit of MINOCYCLINE has been confirmed in another mouse model of familial ALS [47].

MULTIPLE SCLEROSIS is characterized by the infiltration of LEUKOCYTES into the CNS. As MMPs facilitate the passage of LEUKOCYTES across matrix barriers, the hypothesis that targeting MMPs could attenuate neuro-INFLAMMATION has been assessed in EAE, an animal model of MULTIPLE SCLEROSIS [48, 49]. MINOCYCLINE inhibits MMP activity, reduces production of MMP-9 and decreases the transmigration of T LYMPHOCYTES across a fibronectin matrix barrier. It is efficacious against both mild and severe EAE in mice. MINOCYCLINE pretreatment delays the course of the disease, and when maximal disease activity occurs in vehicle-treated EAE mice, MINOCYCLINE-treated animals have minimal signs of INFLAMMATION and demyelination in the CNS. MINOCYCLINE has also been proposed to show therapeutic potential in Alzheimer's disease [50].

Japanese encephalitis (JE), caused by a neurotropic flavivirus, leads to INFLAMMATION in the CNS and neuronal death, and also compromises the structural and functional integrity of the blood-brain barrier (BBB). MINOCYCLINE confers complete protection in mice following JEV infection. Neuronal APOPTOSIS, microglial activation, caspase activity, proinflammatory mediators, and viral titer are markedly decreased in MINOCYCLINE-treated JEV-infected mice on day 9 post infection. Intraperitoneal injection of MINOCYCLINE, beginning 24 h post JEV infection,

reduces BBB damage, decreasing expression of iNOS, cyclooxygenase (COX)-2, VEGF and reducing the level of transcript of chemokine receptors and adhesion molecules in the brain [51].

Congenital muscular dystrophy type 1A is an autosomal recessive disease that is caused by loss-of-function mutations in the laminin-alpha2 gene, and results in motor nerve and skeletal muscle dysfunction. Minocycline (or doxycycline) increases the median lifespan of laminin-α2-null mice and may be a possible route to improving neuromuscular dysfunction caused by laminin-α2 deficiency [52].

Proteolytic degradation of the aortic wall by MMPs is considered important in the pathogenesis of AAA, and tetracyclines, by virtue of their MMP-inhibiting effect, have been proposed as a novel therapeutic strategy. An *ex vivo* study of AAA patients receiving doxycycline before surgery showed a 2.5-fold reduction in MMP-9 and a 5.5-fold reduction in MMP-9 mRNA in aneurysm tissue compared to untreated patients. In addition, exposure to doxycycline also reduced MMP-9 protein and mRNA expression in THP-1 monocytes [53]. However, MMPs play a pivotal role in protecting against pulmonary remodeling, and doxycycline-treated rats in chronic hypoxic conditions (10% O2 for 15 days) had higher pulmonary artery pressure and more severe right ventricular failure than controls [54].

In models of dextran sulfate sodium (DSS)- and trinitrobenzene sulfonic acid (TNBS)-induced acute and chronic colitis, prophylactic and therapeutic treatment of mice with minocycline significantly diminishes mortality rate and attenuates the severity of colitis probably *via* inhibition of iNOS and MMP expression in intestinal tissues [55].

Minocycline was first thought to be a causative agent of possible drug-related connective tissue disease in 1992, and several cases have been reported in the literature. Most cases occurred in young women being treated for acne. The pathophysiological mechanism of this minocycline-induced autoimmune syndrome remains unknown. Mechanisms suggested include hapten formation, unmasking of neoantigens, molecular mimicry, cross-reactivity with self-antigens such as microsomal cytochromes, or unmasking of an underlying lupus diathesis. Factors such as low acetylator status also may play a role in the pathogenesis [56]. A substantial proportion of children with minocycline-induced autoimmunity develop chronic symptoms. Serious adverse effects include hypersensitivity reactions (serum sickness, hypersensitivity pneumonitis, pericarditis) and various autoimmune phenomena (autoimmune hepatitis, drug-induced lupus, arthritis, and vasculitis), development of autoantibodies (antinuclear antibody, antineutrophil cytoplasmic antibody and antiphospholipid antibodies) [57]. Minocycline-associated drug hypersensitivity syndrome may be associated with multiple autoimmune sequelae, including thyroid disease, type 1 diabetes mellitus, and elevated markers of systemic autoimmunity [58].

Chemically modified tetracycline

Up to now, eight different CMTs have been described [59]. Their mechanism of action is still not completely clarified, but they are probably able to down-regulate MMP expression, inhibit pro-MMP activation or directly inhibit active MMPs. Several authors have investigated the inhibitory effects of CMTs on MMP activity *in vitro* and report CMT-3 (Fig. 2), under development by CollaGenex, as one of the most potent CMTs [60]. CMT-3 can reduce the production of proinflammatory mediators (IL-1β, IL-6, IL-8, MMP-8, and MMP-9) by whole blood. In the model of sepsis induced in rats by cecal ligation and puncture, CMT-3 prevents the plasma increase in TNF-α and liver increase in p38 MAPK and p42/44 MAPK. It also inhibits MMP-9, which in turn decreases TGF-β1 and caspase-3 signaling pathways and improves survival in septic rats. The antiproteolytic activity of CMTs may be responsible for their capacity to inhibit the invasiveness, *in vitro* or *in vivo*, of many neoplasms including glioblastoma, tumors of the breast, prostate, and colon, and melanoma. Additionally, the capacity of CMTs to induce apoptosis in a variety of cells (macrophages, tumor cell lines, leukemia cell lines, and prostate tumor cell lines) has been proposed as a basis for their antitumor activity.

Tigecycline

Tigecycline (Fig. 2) is the first clinically available drug in a new class of antibiotics called the glycyl-

cyclines. It is structurally similar to the tetracyclines with a central four-ring carbocyclic skeleton, and is actually a derivative of MINOCYCLINE. Tigecycline has a substitution at the D-9 position to confer broad spectrum antibacterial activity. In a murine model of *Mycoplasma pneumoniae* pneumonia, tigecycline treatment demonstrates a modest microbiological effect, but it significantly improves histological evidence of lung INFLAMMATION and reduces pulmonary CYTOKINES and CHEMOKINES [61].

Fosfomycin

Fosfomycin (1-cis-1,2-epoxypropylphosphoric acid) is a broad-spectrum bactericidal antibiotic that interferes with bacterial cell wall biosynthesis by inhibiting pyruvate-uridine-diphosphate-*N*-acetylglucosamine transferase. It is passively (twofold) accumulated by PMN. *In vitro*, fosfomycin increases PMN bactericidal activity, extracellular oxidant production and intracellular Ca^{2+} concentrations. By contrast, other authors have noted an inhibitory effect of fosfomycin on PMA-stimulated oxidant production by PMN, suggesting an effect on PKC-dependent activation pathways.

Fosfomycin has immunomodulatory activity on B and T LYMPHOCYTE functions, increases SENSITIVITY to TGF-β and inhibits HISTAMINE release from BASOPHILS. Fosfomycin decreases TNF-α and IL-1 synthesis, but increases that of IL-6. The inhibitory effect of fosfomycin on proinflammatory cytokine production seems to be related to inhibition of NF-κB activation [62]. *In vitro*, the addition of fosfomycin to whole human blood incubated with ENDOTOXIN, results in decreases in mRNA levels and release of proinflammatory CYTOKINES [63].

The immunomodulatory activity of fosfomycin (and of its enantiomer, which lacks antimicrobial activity) has been demonstrated in various animal models. Fosfomycin and its enantiomer significantly increase the survival rate in a model of gut-derived *P. aeruginosa* sepsis and reduce serum levels of TNF-α, IL-1 and IL-6. In mice injected with LPS, fosfomycin significantly lowers peak serum levels of TNF-α and IL-1β. In a rat air-pouch model, after carrageenan challenge, fosfomycin decreases the amount of PROSTAGLANDIN E_2 (PGE_2), TNF-α and mRNA encoding COX-2 [64]. The therapeutic benefit of fosfomycin has been assessed in an animal model of Sjögren's syndrome [65].

Fusidic acid

Fusidic acid, a tetracyclic triterpenoic structure used mainly as an anti-staphyloccocal agent, interferes with protein biosynthesis. It decreases PMN functions *in vitro*, without markedly altering monocyte functions. Fusidic acid protects mice from LPS- and staphylococcal enterotoxin B-induced lethality, and suppresses TNF-α and IFN-γ release *in vivo*. Prophylactic administration of fusidin significantly increases the survival of neonatal mice challenged with *Salmonella enteritidis* LPS, and also reduces peak plasma values of TNF-α. The potential immunomodulatory effect of fusidic acid has also been demonstrated in a model of Con A-induced liver damage. Prophylactic administration of fusidic acid protected mice from ConA-induced hepatitis, and this was accompanied by markedly diminished plasma levels of IL-2, IFN-γ and TNF-α, along with increased levels of IL-6. Fusidic acid has proved beneficial in the treatment of experimental autoimmune neuritis in rats (a model of Guillain-Barré syndrome).

Gyrase B inhibitors

Novobiocin and coumermycin impair bacterial DNA replication by inhibiting gyrase B activity. Few studies on immune responses have been done with these compounds. At therapeutic concentrations, coumermycin impairs PMN CHEMOTAXIS, SUPEROXIDE ANION production and intracellular killing. Novobiocin interferes with metabolic processes in eukaryotic cells and is a potent inhibitor of ADP ribosylation. Novobiocin effectively suppresses the production of proinflammatory CYTOKINES (IL-1, IL-6) and the anti-inflammatory cytokine IL-10 by LPS-stimulated MONOCYTES. It also induces CD14 shedding and modulates the expression of other surface ANTIGENS. No immune modulating effects have been demonstrated in animal models.

Isoniazid

Isoniazid, an isonicotinic acid hydrazide, is an antituberculous agent. Its anti-mycobacterial activity has been attributed to its oxidative metabolism by mycobacterial peroxidases. This chemical reactivity explains its inhibition of the myeloperoxidase (MPO)-H_2O_2-halide system and also its potential toxicity after oxidization by activated LEUKOCYTES. Drug-induced lupus is a well-known complication of isoniazid therapy.

Lincosamides

Lincomycin and clindamycin interact with bacterial protein synthesis at the level of the 50S ribosomal subunit. The nucleoside transport system has been suggested to explain the cellular accumulation of clindamycin (12–20-fold). Clindamycin was presented as a possible immunomodulator in infection in the early 1980s. However, controversial effects on phagocyte functions (stimulation, inhibition or no action) have been reported with various techniques and drug concentrations. Interest in this drug was stimulated by its potential prophylactic effect in LPS-induced septic SHOCK, through inhibition of proinflammatory cytokine release *in vitro* and *in vivo* [66]. Interestingly, modulation of cytokine release *in vitro* is not accompanied by a parallel change in mRNA expression [67]. In a canine model of *Babesia gibsoni* infection, clindamycin damages (but does not eliminate) the parasite, stimulating efficient humoral and cellular immune responses and improving clinical outcome [68].

Macrolides

Macrolide antibiotics have a 12–16-membered macrocyclic lactone nucleus, with few double-bonds, substituted usually by two amino and/or neutral sugars. Modern therapeutic agents, and particularly semi-SYNTHETIC derivatives of erythromycin A (Fig. 2) (roxithromycin, clarithromycin, azithromycin), have been obtained by adding new substituents, by introducing a nitrogen atom into the lactone ring (azalides), or, more recently, by removing the l-cladinose at position 3 of the lactone ring and oxidization into a 3-keto function (ketolides). Macrolides impair bacterial protein synthesis by acting on the 50S bacterial ribosomal subunit. Extensive assessment of macrolides has been carried out in the context of INFLAMMATION and cancer.

In vitro effects

All macrolide antibiotics are able to concentrate (10–300-fold) within host cells (particularly PHAGOCYTES) [6]. This property contributes to BIOACTIVITY against facultative and obligate intracellular pathogens. Many data point to the existence of an active transport system in PMN and other mature phagocytic cells. There is some evidence of a link between the P-gP family and the macrolide CARRIER. Cellular accumulation of macrolides is probably responsible for their impact on host cell functions. Structure-activity studies have shown that only erythromycin A derivatives, including the azalide azithromycin, impair the phagocyte oxidative burst in a time- and concentration-dependent manner and directly stimulate exocytosis by human NEUTROPHILS. The chemical entity responsible for these effects is the l-cladinose at position 3 of the lactone ring, but other structures may also interfere with phagocytic transduction targets [69]. The transduction pathway by which erythromycin A derivatives interfere with NEUTROPHILS seems to be the phospholipase D-phosphatidate phosphohydrolase (PLD-PPH) pathway, which is crucial for the activation of exocytosis and oxidant production. Macrolides, including ketolides [70], decrease proinflammatory cytokine production by stimulated PHAGOCYTES, while increasing that of the anti-inflammatory cytokine, IL-10. Individual susceptibility to the immunomodulatory activity of macrolides has been shown for both cytokine production and oxidant production. The underlying mechanism (antibiotic uptake, cellular TARGET, etc.) is not known. Modulation of proinflammatory cytokine production has also been observed in EOSINOPHILS, LYMPHOCYTES and non-phagocytic cells, including normal and transformed human bronchial cells, nasal epithelial cells from polyps of patients with chronic sinusitis, and a lung fibroblast cell

line. In general, the suppression of cytokine release is accompanied by a parallel decrease in mRNA expression. Depending on the cell type, inhibition of various transcription factors (AP-1, NF-κB and NF-AT) has been described [71–73]. Abeyama and colleagues [74] have proposed that modulation of cytokine production is linked to macrolide-induced impairment of the oxidative response.

In a cystic fibrosis (CF) cell line, azithromycin causes a 30% reduction in TNF-α mRNA levels and a 45% decrease in TNF-α secretion, reaching approximately the levels of the untreated isogenic non-CF cells, probably because of inhibition of NF-κB and Sp1 DNA binding [75]. Interestingly, in airway epithelial cells isolated and purified from CF mice, azithromycin rather stimulates basal and LPS-stimulated expression of MIP-2 and KC, two mouse neutrophil chemoattractant mediators that are functionally homologous to human IL-8 [76]. This may explain why, in some CF patients, the effect of azithromycin is ambiguous or inefficient. This ambiguity is reinforced by recent studies from the same group, showing that azithromycin down-regulates inflammatory cytokine production by M1-polarized CF alveolar MACROPHAGES [77], and shifts macrophage polarization towards the alternatively activated phenotype [78].

Other macrolide-induced modifications of mammalian cell functions or metabolism have been reported such as accelerated neutrophil [79] and lymphocyte APOPTOSIS, suppression of ATP-induced Ca^{2+} influx in A549 cells, decreased glycoconjugate secretion by cultured human airway cells, and decreased NOS and COX expression in rat MACROPHAGES. Mucus hypersecretion is a prominent feature in patients with chronic respiratory tract infections such as CF and diffuse panbronchiolitis. Azithromycin and clarithromycin inhibit overproduction of MUC5AC induced by human neutrophil peptide-1 (HNP-1) or LPS stimulation. Telithromycin also has an inhibitory effect on MUC5AC production induced by LPS only [80]. Erythromycin suppresses the gelatinolytic activity of cell-derived MMP-9 and down-regulates the expressions of MMP-9 protein and MMP-9 mRNA in a concentration-dependent manner.

Novel effects have been recently published. Azithromycin increases the transepithelial electrical resistance of human airway epithelia by changing the processing of tight junction proteins [81], and clarithromycin or roxithromycin suppress the antigen-specific immune responses of DENDRITIC CELLS *in vitro* [82].

The pleiotropic effects of macrolides raise the possibility that several common cellular targets of macrolide action exist. Among the cellular mechanisms advocated, inhibition of MAP kinase (e.g., ERK1/2) activity and suppression of transcription factor activation have been suggested [71–73, 83–85].

Modifications of cell functions may also result from modulation of GENE EXPRESSION. For instance, clarithromycin inhibits the expression of the TGF-β, TNF-α, and MMP-9 genes. Erythromycin, clarithromycin, but not josamycin, alter GENE EXPRESSION profiles in long-term-cultured small airway epithelial cells [86].

Animal models

Various animal models have been used to explore the immune mechanisms underlying macrolide actions. Aseptic models (surgical trauma, carrageenin-induced pleurisy, extrinsic allergic alveolitis, LPS inhalation, etc.) have proven useful for the analysis of the anti-inflammatory properties of macrolides. In a model of bleomycin-induced acute lung injury with subsequent fibrosis, 14-membered macrolides attenuate the migration of inflammatory cells into the lung, decrease lung injury and fibrosis, inhibiting or attenuating mRNA expression of the CELL ADHESION MOLECULES VCAM-1 and ICAM-1 [87]. After intranasal LPS challenge, azithromycin or clarithromycin pretreatment reduces total cell and neutrophil numbers in bronchoalveolar lavage fluid and MPO concentration in lung tissue, possibly through inhibition of GM-CSF and IL-1β production by alveolar MACROPHAGES [88].

Serum amyloid A protein (SAA) belongs to a family of acute phase proteins, which are produced in response to infection and inflammatory stimuli. After a sterile inflammatory challenge, azithromycin, clarithromycin and roxithromycin inhibit circulating SAA, with subtle differences as regards to the chronology of the inhibition [89]. IL-10-deficient mice are well characterized as an experimental model of inflammatory bowel disease. Long-term administra-

tion of roxithromycin suppresses the development of severe colitis and decreases the production of IFN-γ and IL-12 [90]. In murine cardiac ALLOGRAFT recipients, clarithromycin improves acute and CHRONIC REJECTION judged by graft survival and by myocardial cell infiltrating area, in a total allomismatch combination, through the suppression of MMP-9 [91].

Inflammatory and infectious models have also been used to demonstrate the immunomodulatory effects of macrolides. In *S. aureus*-induced osteomyelitis, roxithromycin suppresses local expression of IL-1β and TNF-α, without modifying bacterial counts [92]. Azithromycin enhances bacterial CLEARANCE and reduces lung INFLAMMATION by improving innate immune defense mechanisms in CF mice infected with mucoid *P. aeruginosa* [93].

Although the PRECISE mechanisms have not yet been clarified, 14-membered ring macrolides and their derivatives are promising in therapeutic applications for solid tumors. Erythromycin A derivatives increase the survival of tumor-bearing mice (in both allogeneic and syngeneic systems), retard tumor growth, and inhibit metastasis. In a rat model of transplanted mammary adenocarcinoma, clarithromycin displays a synergistic effect with cyclophosphamide or surgery to reduce mortality. Spleen cells from clarithromycin-treated tumor-bearing rats exhibit greater tumor-neutralizing activity and lower IL-6 and TGF-β GENE EXPRESSION than cells from control rats. Roxithromycin and clarithromycin potentiate the antitumor effects of cyclophosphamide, adriamycin and vindesine *in vivo*, without altering their direct CYTOTOXICITY *in vitro*. Inhibitory effects of roxithromycin and clarithromycin on tumor growth and lung metastasis, together with antiangiogenic activity, have also been demonstrated, whereas azithromycin and josamycin are not effective in these systems. However, clarithromycin failed to alter metastatic development of two different human non-small-cell lung cancers in severe combined immunodeficient (SCID) mice. In the model of diethylnitrosamine-induced liver injury with sequential formation of cirrhosis and hepatocellular carcinoma, roxithromycin administration inhibited oxidative stress, NF-κB activation, and iNOS activity, and reduced tumor formation in the liver [94]. It also inhibited angiogenesis of human hepatoma cells *in vivo* by suppressing VEGF production [95].

Human *ex vivo* studies

In general, short-term macrolide treatment enhances immune responses, but long-term exposure results in gradual inhibition. In subjects with chronic obstructive pulmonary disease (COPD), short-term azithromycin therapy causes a transient early increase in the blood neutrophil oxidative burst followed by a decrease in inflammatory markers on longer administration.

Various erythromycin A derivatives have proven beneficial in diffuse panbronchiolitis (DPB) and cystic fibrosis (see section *Therapeutic implications*), and modulation of various inflammatory parameters has been observed in patients receiving macrolide therapy. In DPB patients, neutrophil infiltration and IL-8, LTB4 and elastase levels in bronchoalveolar lavage (BAL) fluid fall in parallel with clinical improvement during erythromycin A therapy. The benefit of azithromycin therapy in CF patients does not seem to involve up-regulation of the multidrug resistance (MDR) or CFTR proteins, or correction of epithelial ion transport and bacterial ADHERENCE [96].

In ASTHMA, inhibition of eosinophil or neutrophil activation has been forwarded to explain the reduction in airway hyperresponsiveness during therapy, and lymphocyte APOPTOSIS has also been reported. Elevated levels of NO in nasal fluid and serum, and increased spontaneous or antigen-stimulated NO production by mononuclear cells from patients with allergic rhinitis, sinusitis and ASTHMA have been observed. Macrolides can decrease NO levels in these patients. In patients with sinusitis, clarithromycin and roxithromycin modulate PMN expression of L-selectin and Mac-1, thereby attenuating PMN adhesiveness. In addition, MACROPHAGES isolated from nasal polyps of macrolide-treated patients express more CD80 (a COSTIMULATORY MOLECULE) than control cells, and this correlates negatively with the number of EOSINOPHILS infiltrating the polyps.

EM703

EM703 (Fig. 2), an erythromycin derivative synthesized by the group of Omura (Kitasato institute), shows no antibacterial action, but has potent immu-

nomodulatory activity. In human bronchial epithelial cells, EM703 suppresses the activation of NF-κB and the production of IL-8.

In the model of bleomycin-induced lung fibrosis, the infiltration of MACROPHAGES and NEUTROPHILS into the airspace, fibroblast proliferation and collagen production are inhibited by EM703 [97]. EM703 inhibits also the transcription of type I collagen in both normal and systemic sclerosis fibroblasts [98], and TGF-β signaling in human lung fibroblasts [99].

Other erythromycin derivatives are under development as potential inhibitors of residual HIV-1 in the lymphoreticular system of HIV-1-infected patients and offer great promise for the creation of new anti-HIV drugs [100]. EM201 and EM703 inhibit the replication of HIV-1 in tissue MACROPHAGES, at post-transcriptional and translational levels. They convert tissue MACROPHAGES from HIV-1-susceptible to HIV-1-resistant cells through down-regulation of Hck (hematopoietic cell kinase) and induction of small isoforms of CCAAT enhancer binding protein β (C/EBPβ). These drugs inhibit p38 MAPK activation, which is expressed only in susceptible tissue MACROPHAGES. Activated $CD4^+$ T cells stimulate the viral replication in HIV-1-resistant MACROPHAGES through down-regulation of small isoforms of C/EBPβ *via* activation of ERK1/2. EM201 and EM703 inhibit MAPK activation and the following burst of viral replication.

Peptides

Peptide antibiotics comprise polypeptides (tyrocidins, gramicidins and bacitracin), polymyxins, streptogramins, glycopeptides (vancomycin, oritavancin), the lipopeptide daptomycin and the lipoglycopeptide teicoplanin. The mechanisms underlying the antibacterial activity of these drugs differ. Polymyxins act by increasing the permeability of the cytoplasmic membrane, while glycopeptides interfere with cell wall biosynthesis and streptogramins impair bacterial protein biosynthesis by acting on the 50S ribosomal subunit. Cellular uptake of these drugs also varies. Daptomycin shows poor cellular accumulation (0.6-fold), teicoplanin (mainly membrane-bound) 50-fold (like streptogramins), and oritavancin more than 300-fold [101].

In general, peptide antibiotics do not significantly alter immune functions at therapeutic concentrations. The drug most extensively studied in this respect is polymyxin B, one of the first recognized inhibitors of protein kinase C. Polymyxin B has a stimulatory effect on monocyte function, stimulating the production of IL-1, IL-6, GM-CSF and COMPLEMENT COMPONENTS. The capacity of polymyxin B to bind the lipid A portion of LPS is unfortunately associated with toxicity, ruling out its general use in septic SHOCK. Bacitracin binds Ca^{2+} and Mg^{2+}, a property that has been held responsible for the inhibitory effect of this drug on PHAGOCYTOSIS. Colistin increases the activity of human neutrophil elastase and *P. aeruginosa* elastase, two proteases that contribute to the pathogenesis of CF. Vancomycin and teicoplanin depress some PMN functions, but only at very high, clinically irrelevant concentrations. Vancomycin can induce NEUTROPENIA and anaphylactoid reactions and promotes HISTAMINE release from rat peritoneal MAST CELLS *in vitro*. At a concentration of 50 mg/L, teicoplanin also increases the production of TNF-α, IL-1 and IL-6 by conA-stimulated human MONOCYTES.

Quinolones

Quinolones are SYNTHETIC antibacterial compounds whose first representative (nalidixic acid) was synthesized in 1962. Thousands of compounds have been made, of which the 6-fluorinated molecules (fluoroquinolones) represent a breakthrough in quinolone research (Fig. 2). The antibacterial activity of quinolones stems from their inhibitory effect on bacterial DNA gyrase (topoisomerase II), and thus on DNA replication. Quinolones might also affect mammalian DNA metabolism, as mammalian cells also contain an essential type II DNA topoisomerase. Fluoroquinolones modify immune and inflammatory responses *in vitro* and affect *in vivo* cellular and humoral immunity by attenuating cytokine responses [102].

In vitro effect

Most quinolones seem to enter and exit from loaded PHAGOCYTES by passive diffusion, although an

unidentified active transporter has been proposed for pefloxacin and ciprofloxacin. Some quinolones, such as difloxacin, ciprofloxacin and ofloxacin, may use a P-gp-like RECEPTOR as efflux CARRIER. Cellular (mainly cytosolic) concentrations are about 4–10-fold higher than extracellular concentrations, except for grepafloxacin (about 66-fold). Significant intracellular bactericidal synergy with oxidants has been reported with some molecules, and a synergistic effect between G-CSF and ofloxacin on PMN bactericidal activity has been observed. The underlying mechanisms (G-CSF-induced increase in ofloxacin uptake or in SUPEROXIDE ANION production) were not investigated.

At therapeutic concentrations, quinolones differently affect PHAGOCYTOSIS, adhesion, and oxidant production by rat peritoneal MACROPHAGES and human PMN. Their effects (increase, decrease, no effect) on oxidant production appear to depend on the animal species and the quinolone structure. Ofloxacin induces an increase in the PMN oxidative response by enhancement of PKC activity, whereas norfloxacin increases oxidant production by mouse MACROPHAGES through enhanced mobilization of NADPH OXIDASE subunits. All quinolones modestly but significantly impair rat macrophage CHEMOTAXIS, in a concentration-dependent manner. The effects of quinolones on cytokine production by MONOCYTES have been widely documented [102]. At high concentrations, pefloxacin and ciprofloxacin decrease IL-1 production by LPS-stimulated human MONOCYTES, and ciprofloxacin and ofloxacin (>25 mg/L) decrease TNF-α production. These depressive effects may be linked to CYCLIC AMP accumulation. A suppressive effect of therapeutically achievable trovafloxacin concentrations on the synthesis of IL-1α, and β, IL-6, IL-10, GM-CSF and TNF-α by LPS-stimulated human MONOCYTES has also been reported. Similar results have been obtained with moxifloxacin. Grepafloxacin inhibits the production of IL-1α and β and the expression of IL-1α and β, IL-6 and IL-8 mRNA, suggesting an effect at the gene transcription level. Ciprofloxacin decreases IL-6 mRNA and increases IL-8 mRNA expression but does not modulate activation of NF-κB or AP-1 [103]. LPS-stimulated release of pre-synthesized IL-1β is promoted by levofloxacin, in part *via* the p38 MAPK pathway but newly synthesized IL-1β production is inhibited at the post-transcriptional level [104].

The effects of grepafloxacin and moxifloxacin on the release of CYTOKINES, chemical mediators, hydrolytic enzyme activities, and lipoxygenation have been evaluated in zymogen A- or *S. aureus*-stimulated human THP-1 MONOCYTES [105, 106]. Within the first hour, the release of CYTOKINES (TNF-α, IL-1, IL-6, and IL-8), chemical oxidants (NO and H_2O_2) and hydrolytic enzymes are increased. A second phase between 2–4 hours is characterized by the suppression of mediators involved in INFLAMMATION. The third response, an apparent bacteriostatic inhibition of DNA synthesis, causes bacterial death. The quinolones appear initially to activate MONOCYTES to kill bacteria through the innate immune process by releasing oxidants and lysosomal hydrolytic enzymes. At a later time, the bacteria are killed and there is a reversal of the effects of quinolones on cytokine release, FREE RADICAL generation and hydrolytic enzymes so that lipid peroxidation and tissue destruction by the infection process is suppressed.

Several authors have observed that quinolones alter T and B lymphocyte functions and delay or suppress the proliferative response of human mononuclear cells. Most derivatives superinduce IL-2 synthesis and, to a lesser extent, IFN-γ synthesis, by MITOGEN-activated LYMPHOCYTES. Lymphokine mRNA profiles are also up-regulated, suggesting a mammalian stress and/or DNA damage response. The activity of transcriptional regulatory factors (NFAT-1 and AP-1) is increased. IL-8 production and E-SELECTIN expression by human endothelial cells are increased by trovafloxacin.

In vivo/ex vivo effects

The potential value of quinolones as anti-inflammatory agents has been proposed, based on their modulation of cytokine responses. Accordingly, several animal models have been studied. Trovafloxacin, ciprofloxacin and tosufloxacin significantly protect mice injected with a lethal dose of LPS and diminish serum levels of IL-6. A beneficial effect of ciprofloxacin, rufloxacin, difloxacin, trovafloxacin and temafloxacin has also been observed on *Bacteroides fragilis* (a resistant pathogen)-induced intra-abdominal abscesses. The protective effect was related to a decrease in TNF-α production. In TNBS (2,4,6-trini-

trobenzene sulfonic acid)-induced colitis in BALB/c mice, there is a significant decrease of IL-1β, IL-8, and TNF-α levels in colon homogenates of ciprofloxacin-treated animals [107].

The effect of oral ofloxacin prophylaxis on ENDOTOXIN/cytokine release in aortic aneurysm repair has been evaluated in 25 patients with infrarenal aortic aneurysm [108]. Ofloxacin had no effect on the occurrence of complications or on the peripheral ENDOTOXIN levels but ofloxacin-treated patients showed increased ENDOTOXIN neutralizing capacity and increased IL-6 levels preoperatively and at 30 minutes after clamping. Ciprofloxacin may have an immunomodulatory effect on septic patients by attenuating the proinflammatory response, but there is no evidence that differences in the CYTOKINES measured have any impact on the final outcome [109].

To date, quinolones have not proven beneficial in inflammatory diseases. Interest in the potential immunostimulating properties of some fluoroquinolones is growing. Ciprofloxacin and moxifloxacin demonstrate a beneficial effect on HEMATOPOIESIS. Accelerated neutrophil recovery has also been obtained after ciprofloxacin administration to BONE MARROW-transplanted patients and patients suffering from breast cancer under chemotherapy.

Riminophenazines

In structural terms, riminophenazines are phenazine compounds in which a substituent (R) is included in the "imino" part of the molecule. The first compound developed for clinical use was clofazimine (Lamprene), in mycobacterial diseases. The anti-mycobacterial mechanism of these drugs has not yet been clarified. Intracellular (phagocytic) accumulation of riminophenazines is a key factor in their BIOACTIVITY against mycobacteria, which are obligate intracellular pathogens. This intracellular activity is potentiated by phagocyte treatment with IFN-γ or TNF-α. Clofazimine increases SUPEROXIDE ANION production and DEGRANULATION by stimulated NEUTROPHILS, and TNF-α potentiates this enhancement. The mechanism underlying this pro-oxidative effect seems to involve stimulation of phospholipase A_2 (PLA_2) activity, with subsequent accumulation of arachidonic acid and lysophospholipids, which act as second messengers to activate the oxidase. In addition, PLA_2 activation and lysophosphotidylcholine accumulation have been held responsible for inhibition of the membrane Na^+, K^+-ATPase, a key enzyme in various lymphocyte functions. CICLOSPORIN potentiates the immunosuppressive activity of clofazimine through a PLA_2-Na^+, K^+-ATPase-dependent mechanism. Other immunosuppressive/anti-inflammatory effects of clofazimine could be related to its capacity to scavenge chlorinating oxidants and to stimulate PGE_2 production by NEUTROPHILS.

Sulfones/sulfonamides

Dapsone (4,4' diaminophenyl sulfone) was initially developed as an anti-tubercular drug. It was tested in leprosy in the early 1950s and is still a part of drug combinations used in this disease. It was later tested in malaria and some inflammatory diseases. Its antibacterial activity is due to inhibition of dihydropteroate synthase. The anti-inflammatory activity of dapsone is less well understood. Dapsone inhibits neutrophil functions such as CHEMOTAXIS and oxidant production. It also impairs neutrophil ADHERENCE to ANTIBODIES bound to the basement membrane (probably by direct interference with the ANTIBODIES). In addition, it irreversibly inhibits MPO, by converting the enzyme into its inactive (ferryl) form. The hematological toxicity of dapsone is linked to its oxidative metabolism.

Dapsone impairs the production of PGE_2 by NEUTROPHILS, possibly explaining dapsone-induced potentiation of CELL-MEDIATED IMMUNITY. It suppresses mRNA expression of TNF-α and significantly decreases the level of TNF-α in culture supernatants of LPS-stimulated PMBC. Dapsone can bring about significant clinical improvement in patients with cutaneous lupus erythematosus.

Sulfonamides also inhibit dihydropteroate synthase. The most frequently used antibacterial sulfamide is sulfamethoxazole, in combination with trimethoprim (cotrimoxazole). In general, sulfonamides inhibit phagocyte functions, and many agents in this class have been switched from infections to anti-inflammatory indications. The mechanisms

underlying these immunomodulatory effects are unclear. Inhibition of the elevation of intracellular Ca^{2+} after stimulation has been reported with SULFASALAZINE and sulfapyridine. Sulfapyridine, but not sulfamethoxazole, has been reported to scavenge HOCl.

Cutaneous drug reactions are the most common adverse drug reactions associated with sulfamethoxazole and dapsone. Keratinocytes bioactivate these drugs to reactive arylhydroxylamine metabolites and form detectable covalent adducts with cellular macromolecules. Normal human epidermal keratinocytes selectively up-regulate certain danger signals when exposed to arylhydroxylamine metabolites. These signals may subsequently activate DENDRITIC CELLS and initiate an immune response within the skin [110].

Other antibiotics

Some authors have studied the *in vitro* effects of ethambutol, nitrofurans and minimally substituted imidazoles (metronidazole and tinidazole) on immune cell functions, but no significant alterations have been found. Few data are available on the recently introduced oxazolidinone class. Linezolid at therapeutic or supratherapeutic concentrations does not influence human PMN function. Various oxazolidinone derivatives such as locostatin are promising agents to control INFLAMMATION, sepsis, and AUTOIMMUNE DISEASES [111].

Non-antibacterial effects of antibacterial agents: Therapeutic implications

The therapeutic relevance of the immunomodulatory actions of antibacterial agents is controversial, and there is no general agreement on whether these effects must be taken into account when choosing an antibacterial treatment. The clinical benefit of the immunostimulating/restoring effects of antibacterial agents is considered minimal compared to their direct antibacterial activity. By contrast, some antibacterials with immunodepressive potential are showing promise in inflammatory diseases, particularly in the therapy of respiratory tract infections [112, 113].

Immunostimulating antibacterial agents

In the 1990s, cefodizime was presented as a forerunner IMMUNE RESPONSE MODIFIER antibiotic with both classical antibacterial activity and innovative immunomodulatory potential. Despite the abundance of published data, the development of cefodizime as an immunomodulatory antibiotic has been unsuccessful. No reports are available on the consequences of prophylactic administration of cefodizime in patients at risk of infections.

Immunodepressive antibacterial agents

The use of antibacterial agents in inflammatory diseases has been supported by two hypotheses. Either microorganisms can initiate an excessive and/or chronic inflammatory reaction, and ABA down-regulate INFLAMMATION by suppressing its bacterial origin; or ABA directly affect the IMMUNE SYSTEM and modulate the inflammatory response (or correct an immunological dysfunction). Various ABA are used in inflammatory diseases (Tab. 5). Three classes have stimulated widespread interest in the context of inflammatory diseases, namely macrolides, cyclines and ansamycins.

Macrolides

Macrolides display immunomodulatory properties that may confer beneficial effects to patients with respiratory diseases associated with chronic INFLAMMATION [114, 115]. They attenuate inflammatory responses in the lung, regulate mucus production, and decrease bronchial responsiveness. The biological rationale and the available clinical data on chronic macrolide therapy in chronic respiratory tract diseases, together with the presumed

mechanisms underlying clinical benefits have been reviewed [116]. Diffuse panbronchiolitis (DPB) and cystic fibrosis are the two main clinical indications for macrolide action [117–119].

Treatment of acute ASTHMA exacerbations is a major need not adequately met by current therapies. Although virus infections are the major cause of acute exacerbations, other factors can increase the risk/severity of exacerbations. In patients with ASTHMA, macrolides reduce airway hyperresponsiveness and improve pulmonary function, and were historically selected for their steroid-sparing effect. Controlled studies have reported small improvements in lung function with macrolide treatment of stable ASTHMA [120]. The TELICAST study has shown telithromycin to be effective in the treatment of acute exacerbations of ASTHMA, although the mechanisms were not determined. Eradication of persistent airway infection with *Chlamydia pneumoniae* and *M. pneumoniae* in patients with ASTHMA may also play a role [121].

Chronic obstructive pulmonary disease (COPD) is a leading cause of death and disability worldwide. The Global Burden of Disease study has concluded that COPD will become the third leading cause of death worldwide by 2020, and will increase its ranking of disability-adjusted life years lost from 12th to 5th. Preliminary data from studies of patients with COPD have shown improvements in symptom scores and respiratory function after macrolide treatment. Additional, prospective, controlled data are required to define any potential treatment effect, the nature of this effect, and the role of bronchiectasis, baseline colonization, and other co-morbidities [122].

Bronchiolitis obliterans syndrome (BOS) is a form of chronic ALLOGRAFT dysfunction in lung transplant recipients. A number of recent cohort studies have given encouraging results with macrolide therapy [123]. Long-term administration with oral azithromycin does not reverse BOS, but may slow progression of the disease [124]. Beneficial effects on gastroesophageal reflux disease may be a mechanism of action [125]. Azithromycin can improve airflow limitation in a significant proportion of patients. The majority of responders were identified after 3 months of treatment. Recent clinical observations, supported by research findings, have revealed a dichotomy in the clinical spectrum of BOS. Neutrophilic (partially) reversible ALLOGRAFT dysfunction responds to azithromycin, while fibroproliferative BOS does not respond to azithromycin [126].

Bronchopulmonary dysplasia (BPD) is a pulmonary disorder that causes significant morbidity and mortality in premature infants. When azithromycin prophylaxis was given to extremely low birth weight infants, mortality, incidence of BPD and other morbidities were not significantly different from control groups except for reduced post-natal steroid use [127].

The use of macrolides in other inflammatory diseases is increasing. Macrolides show promise in various skin diseases such as prurigo pigmentosa, confluent and reticulated papillomatosis, psoriasis and possibly, linear IgA disease and the related chronic immunobullous disease of childhood. Long-term treatment with azithromycin has been used successfully in the SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis) syndrome.

The use of antibiotics in coronary artery diseases (CAD) was initially based on the hypothesis of an infection (*C. pneumoniae*)-linked etiology. However, the possibility of a direct anti-inflammatory action of macrolides in such settings has been advocated by several investigators. The early promise that macrolides may be of benefit to patients who had experienced an acute coronary event, does not seem to have been fulfilled by prospective trials [128] and controversial results (benefit or no effects) have been obtained [129–134]. The WIZARD trial (with Zithromax against atherosclerotic-related disorders, weekly intervention with Zithromax for atherosclerosis and its related disorders), the largest trial of antibiotic therapy for coronary artery disease, involved 7747 adults enrolled by 271 centers in 9 countries [131]. The results demonstrated no significant risk reduction in the likelihood of a primary event with azithromycin *versus* placebo after a median of 14 months of follow-up. Similarly, a 1-year course of weekly azithromycin did not alter the risk of cardiac events among patients with stable coronary artery disease [132] and roxithromycin given for 6 weeks in patients undergoing percutaneous coronary angioplasty was not associated with a reduction of symptomatic restenoses [133]. A short-term course of

Table 5. Therapeutic indications of ABA in the context of inflammatory diseases

ABA	Therapeutic uses
Dapsone	Neutrophilic dermatoses, dermatitis herpetiformis, leukocytoclastic vasculitis, bullous lupus erythematosus, pustular psoriasis, erythema elevatum diutinum Crohn's disease
Clofazimine	Vitiligo, discoid lupus erythematosus, pyoderma gangrenosum, pustular psoriasis
Sulfonamides	Wegener's granulomatosis
Cyclines	*Antibacterial effects in:* Reactive arthritis? (uroarthritis) Rheumatoid arthritis Periodontal diseases Acne vulgaris, rosacea *Immunomodulatory effects in:* Acne vulgaris, rosacea (doxycycline) Skin diseases: pemphigus vulgaris, foliaceous and bullous pemphigoid Periodontal diseases Reactive arthritis? (uroarthritis) Rheumatoid arthritis (minocycline) Scleroderma (minocycline) control of calcinosis in systemic sclerosis (minocycline) Diabetic nephropathy Chronic airway inflammation (asthma, bronchiectasis, acute respiratory distress syndrome, chemical induced lung damage and cystic fibrosis) Adjunct to antipsychotic medications Abdominal aortic aneurysms (doxycycline) Cancer (chemically-modified tetracycline)
Ansamycins	Controversial: Rheumatoid arthritis (rifampicin) Juvenile pauci/polyarticular rheumatoid arthritis, ankylosing spondylitis (intra-articular rifamycin SV) Crohn's disease? (combined with macrolides), rifaximin Cancer (geldanamycin derivatives)
Macrolides (Erythromycin A and derivatives: roxithromycin, clarithromycin, azithromycin)	*Immunomodulatory effects in:* Diffuse panbronchiolitis, cystic fibrosis, chronic bronchitis, bronchiectasis, chronic obstructive pulmonary disease, Bronchiolitis obliterans syndrome (Bronchopulmonary dysplasia?) Rhinosinusitis, nasal polyposis Skin diseases: prurigo pigmentosa, confluent and reticulated papillomatosis, Psoriasis Chronic recurrent multifocal osteomyelitis (azithromycin) Cancer: non-small-cell lung cancer (clarithromycin) Waldenström's macroglobulinemia, myeloma (Clarithromycin + low dose thalidomide + dexamethasone) *Antibacterial-related effects in:* Hodgkin's sarcoma Asthma

Table 5 *(continued)*

ABA	Therapeutic uses
	Controversial: Crohn's disease? Coronary artery diseases? (azithromycin, roxithromycin) Small abdominal aortic aneurysms ?
Other antibacterial agents (new prospects)	Gentamicin (IV): Cystic fibrosis (class 1 mutation)? (long-term treatment not suitable) Fosfomycin: Inflammation Fusidic acid: Inflammation Quinolones: Neutrophil recovery after chemotherapy? Oxazolidinone derivatives (locostatin): Inflammation, sepsis, and autoimmune diseases

azithromycin offered no benefit for survival or ankle pressure in PAD (stable peripheral arterial disease) patients [134]. However, whereas roxithromycin is beneficial, compared to placebo, on the expansion rate of small AAA [135], azithromycin treatment did not have any effect on AAA expansion [136].

The antibacterial activity of macrolides has been thought to underlie their therapeutic use in Crohn's disease (CD). An open LABEL study showed an impressive response to clarithromycin in a group of patients with active CD, many of whom had been resistant to other therapies. However, in a placebo-controlled clinical trial, clarithromycin (1 g once a day for 3 months) was ineffective in achieving remission in active CD, although a significant response/remission rate was observed at 1 month [137]. Various studies support the concept that intra-macrophage bacteria, particularly *E. coli*, may represent an important therapeutic TARGET in CD, and clarithromycin is probably not the optimal choice of antibiotic in this case.

Other interesting prospects for clarithromycin include its potential benefit in cancer. Most studies have involved Japanese patients with unresectable non-small-cell lung cancer: Clarithromycin (400 mg/day, as long as the patients could tolerate it) significantly increases the survival of patients [138]. The combination of clarithromycin (Biaxin) with low-dose thalidomide and dexamethasone (BLT-D) has been proposed as a salvage regimen in Waldenström's macroglobulinemia [139] and has been used successfully in a phase II study to treat patients with relapsed and refractory myeloma [140]. A regression of pulmonary Hodgkin's disease has been observed after prolonged treatment with ciprofloxacin and clarithromycin, which led the authors to hypothesize a bacterial origin for this disease [141].

Cyclines

The non-antibiotic properties of tetracycline and its analogues and their potential for clinical application have been reviewed [142, 143]. Removal of the dimethylamine group at C4 of the tetracycline molecule reduces its antibiotic properties, enhancing its non-antimicrobial actions.

Tetracyclines have long been considered useful adjuncts in peridontal therapy based on their antimicrobial EFFICACY against putative periodontopathogens. However, inhibition of mammalian collagenases and several other MMPs independently of their antimicrobial activity has been recognized. Acne is a therapeutic TARGET of cyclines. One mechanism by which this drug exerts its effect is by inhibiting the proliferation of *Propionibacterium acnes*. However, the lack of correlation between the drug dose regimen and cutaneous bacterial counts has led to speculation that this drug also interferes with the inflammatory reaction. Acne vulgaris and rosacea present therapeutic challenges due to their

chronicity, potential for disfigurement, and psychosocial impact. Although pathophysiologically distinct, both conditions have major inflammatory components. Sub-antimicrobial dosing of doxycycline proved beneficial in a double-blind, placebo-controlled trial in the treatment of moderate facial acne as well as in an open LABEL study in the treatment of rosacea [144]. Cyclines are also effective in various skin diseases, including immunobullous disorders (pemphigous vulgaris, foliaceous and bullous pemphigoid), with fewer side effects than immunosuppressive drugs, although frequent hyperpigmentation has been observed with MINOCYCLINE.

Another TARGET of cyclines is RA. An infectious etiology such as persistent *Mycoplasma* infections has been forwarded to explain the benefit of lengthy courses of tetracyclines in this disease. The EFFICACY of MINOCYCLINE in RA has been reported in two open trials and in three double-blind controlled studies [145]. In a 2-year, double-blind protocol, the EFFICACY of MINOCYCLINE was compared with that of hydroxychloroquine in patients with early seropositive RA [146]. Patients treated with MINOCYCLINE showed more pronounced improvement of disease and received less prednisone than those receiving hydroxychloroquine and they were more likely to have discontinued treatment with prednisone at 2 years. Tetracyclines have also been used in reactive arthritis, i.e., non-purulent INFLAMMATION of a joint following urogenital, gastrointestinal or lower respiratory tract infections, but they only seem to be effective in the case of uroarthritis. However, Chlamydia is a known trigger of reactive arthritis, and may also be a common cause of undifferentiated spondyloarthropathy, suggesting that cycline activity is related to a strict antibiotic effect. MINOCYCLINE has also been used in early diffuse scleroderma and may be effective in the control of calcinosis. Inhibition of MMPs, anti-inflammatory effects, and calcium-binding properties may play a role in this setting. For cutaneous sarcoidosis refractory to classical therapeutics, a trial of MINOCYCLINE or doxycycline is in order [147].

Eradication of *C. pneumoniae* infection and inhibition of elastolytic MMPS by doxycycline have been suggested to reduce the growth rates of small AAA [148]. A clinical trial evaluated the effect of 2 weeks of low- (50 mg/day), medium- (100 mg/day), or high-dose (300 mg/day) doxycycline *versus* no medication in four groups of 15 patients undergoing elective AAA repair. Independent of its dose, short-term preoperative doxycycline therapy improves the proteolytic balance in AAA, presumably through an effect on aortic wall neutrophil content [149].

The possible benefits of doxycycline in glomerulonephritis in humans have been reported recently [150]. Activity of MMPs contributes to the pathogenesis of diabetic proteinuria and proteinuria in patients with diabetic nephropathy can be reduced with low-dose doxycycline therapy over a 2-month period of drug administration. Further studies are necessary to determine the long-term effect, the optimal dose, and the optimal duration of this potentially novel therapy [151].

Tetracycline and related compounds appear to be beneficial for treatment of several chronic inflammatory airway diseases including ASTHMA, bronchiectasis, acute respiratory distress syndrome, chemical-induced lung damage and CF [152].

Among the tetracycline derivatives, MINOCYCLINE is reported to be the only one that has neuroprotective activity (reviewed in [153]). In a pilot investigation in patients with schizophrenia, MINOCYCLINE appeared to be a safe and effective adjunct to antipsychotic medication [154].

Among other therapeutic prospect for cyclines, a recent trial has assessed the ability of tetracycline to decrease the inflammatory process involving *Helicobacter pylori*-associated gastritis, which is thought to lead to epithelial damage and contribute to the development of gastric cancer [155]. In a 16-week placebo-controlled clinical trial involving 374 *H. pylori*-associated gastritis patients, tetracycline-treated patients showed a reduction in INFLAMMATION and epithelial damage independent of a change in *H. pylori* density. This suggests that tetracycline can decrease INFLAMMATION independently of a reduction in the bacterial load.

The tetracyclines and, particularly, the non-antibiotic chemically modified tetracyclines, interfere with several aspects of MMP expression and activation and inhibit tumor growth and metastases in preclinical models. A representative agent of this class, COL-3, is currently undergoing phase I clinical trials [156]. COL-3 was administered orally once daily at

one of two doses (50 or 100 mg) to patients with AIDS-related Kaposi syndrome. COL-3, 50 mg/day, was both active and well tolerated and may represent a promising agent for the treatment of this opportunistic neoplasm of AIDS [157].

Ansamycins

Some anecdotal reports of RA improving in patients with coexisting tuberculosis treated with rifampicin had suggested a potential application of this drug in this disease. However, various studies in larger groups have failed to confirm the usefulness of rifampicin in the treatment of RA in early stages of disease. Intra-articular rifamycin is effective against active synovitis and can profitably be combined with any basic therapy with slow-acting anti-rheumatic drugs. The therapeutic activity of rifamycin SV administered by the intra-articular route has also been evaluated in children with juvenile RA (oligopolyarthritis).

Another TARGET for ansamycins is CD. Rifaximin, a non-absorbable broad-spectrum antibiotic, may be useful in the treatment of ulcerative colitis and pouchitis, since its absorption through inflamed mucosa is negligible. It maintains a TOPICAL action without systemic effects and the lack of resistant bacterial strains may allow prolonged and repeated treatments.

The ansamycin class has been extensively studied by cancer researchers. Despite its potent antitumor potential, geldanamycin presents several major drawbacks as a drug candidate, including hepatotoxicity. This has led to the development of geldanamycin derivatives, which have entered clinical evaluation. A complete evaluation of these interesting compounds has been provided [158].

Conclusions

After decades of research, interest in the immunomodulatory potential of ABA is still growing. It is widely acknowledged that in addition to their antibacterial activity, many ABA display immunomodulatory properties with potential therapeutic importance. Modulation of immune functions is a major focus of attention, particularly in inflammatory diseases and cancer. Tetracyclines, macrolides and to some extent, ansamycins, all show inhibitory activity towards several initiators of the inflammatory cascade, as well as to mediators of tissue damage. However, lengthy administration and absence of selectivity of these antimicrobial immunomodulators can lead to the induction of microbial resistance. Intensive research is ongoing to identify immunomodulatory antibiotic derivatives that are devoid of antibacterial activity, most notably with tetracycline and macrolide derivatives.

On the other hand, the etiology of inflammatory disorders involves many cellular, plasma and humoral signaling pathways culminating in the production of enzymatic and FREE RADICAL-mediated tissue damage. For many years, ROS were classically described as harmful by-products of aerobic metabolism. However, besides a role in phagocyte function and host defense, a large amount of evidence points to important roles for ROS in cell proliferation, APOPTOSIS, angiogenesis and endocrine-related functions and new data argue for a paradoxical benefit of ROS in protecting against arthritis. More intriguing is the fact that enhancing the oxidative burst and ROS production paradoxically ameliorates arthritis [159, 160]. These data may open new strategies in the utilization of antibiotic-induced IMMUNOMODULATION and restore once again to the limelight the "immune-stimulating" antibiotics that have long been pioneers in this saga.

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Cytotoxic drugs

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Background

CYTOTOXIC immunosuppressive agents have a long-standing important role in pharmacological IMMUNOSUPPRESSION. Azathioprine was among the first immunosuppressive drugs used in organ transplantation, and further development in this field was landmarked by the introduction of ALKYLATING AGENTS (i.e., cyclophosphamide) and ANTIMETABOLITES (i.e., fludarabine, METHOTREXATE and mycophenolic acid) in therapeutic regimens for the prevention of graft rejection (see chapter C12) and the treatment of AUTOIMMUNE DISEASES (see chapter C15) because of their well-documented lymphocytolytic effect.

The role of CYTOTOXIC DRUGS is being challenged by steroids, non-steroidal anti-inflammatory agents (NSAIDs), poly- and MONOCLONAL ANTIBODIES, calcineurin and MTOR INHIBITORS (discussed in more detail in chapter C12). However, the reduction of the toxicity burden of IMMUNOSUPPRESSION is currently under investigation with the therapeutic use of CYTOTOXIC DRUGS (i.e., mycophenolic acid) in combination with immunosuppressive ANTIBODIES with the aim of developing calcineurin- and steroid-free immunosuppressive regimens.

Azathioprine

Introduction

Azathioprine, an imidazolyl derivative of 6-mercaptopurine, was developed in the 1950s to improve the BIOAVAILABILITY of its parent drug, mercaptopurine [1]. Animal studies demonstrated that AZATHIOPRINE had a higher THERAPEUTIC INDEX and was a better immunosuppressant than mercaptopurine. Azathioprine is still used in combination regimens with steroids and CALCINEURIN INHIBITORS in patients receiving solid-organ transplants, as well as in AUTOIMMUNE DISEASE therapy in rheumatology, dermatology and gastroenterology [1]. Its use as an immunosuppressive and a corticosteroid-sparing agent is being replaced by mycophenolate mofetil, which is considered a safer and more effective agent, despite the fact that recent findings do not support this evidence [2].

Chemical structure

Azathioprine is an ANTIMETABOLITE prodrug for 6-mercaptopurine, with an imidazolyl group attached to the SH group of 6-mercaptopurine (Fig. 1) to protect it from *in vivo* oxidation. In tissues, AZATHIOPRINE is non-enzymatically converted to mercaptopurine.

Mechanism of action and pharmacological effect

Azathioprine is a CYTOTOXIC ANTIMETABOLITE inhibitor of nucleic acid synthesis. In particular, its metabolite 6-mercaptopurine is further activated intracellularly by ANABOLIC BIOTRANSFORMATION to 6-thioinosinic and 6-thioguanine acid, which interfere with the metabolism of inosine monophosphate (IMP) to adenosine monophosphate (AMP) and triphosphate (ATP), thereby impairing the purine *de novo* biosynthetic pathway. Moreover, active metabolites are incorporated into RNA as well as DNA and its replication is inhibited. The drug suppresses the proliferation of T and B LYMPHOCYTES and reduces the number of CYTOTOXIC T cells and plasma cells in circulation and peripheral organs, thereby decreasing the immuno-

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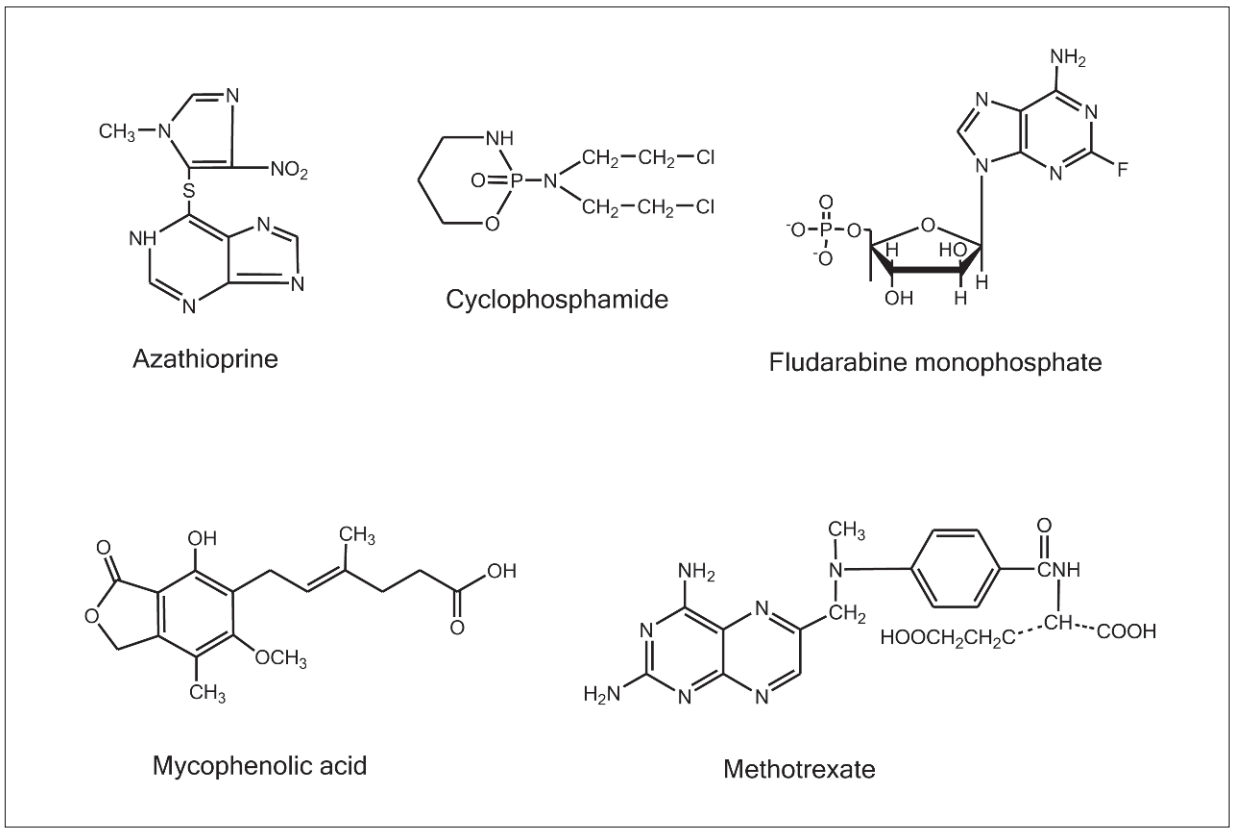

Figure 1. Chemical structures of azathioprine, cyclophosphamide, fludarabine, methotrexate and mycophenolic acid

logical reactivity of the host [3]. For these reasons, AZATHIOPRINE may exert a modest anti-inflammatory effect.

Pharmacokinetics

Azathioprine is rapidly absorbed within 1–2 hours after administration, and evenly distributed in all tissues, although the drug does not cross the blood–brain barrier. Azathioprine is rapidly converted in the liver and ERYTHROCYTES to 6-mercaptopurine and S-methyl-4-nitro-5-thioimidazole by sulfhydryl-containing compounds. Although it is generally recognized that 6-mercaptopurine is the active drug, a previous study has suggested that the imidazolyl moiety might have immunosuppressive activity on its own [4]. After standard oral doses, the terminal half-lives ($t_{1/2}$) of AZATHIOPRINE and 6-mercaptopurine are 50 and 74 minutes, respectively. Azathioprine is mainly metabolized by xanthine oxidase, followed by thiopurine methyltransferase (TPMT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT). 6-Mercaptopurine is inactivated by xanthine oxidase to 6-thiouric acid and by the widely distributed enzyme TPMT to 6-methylmercaptopurine, with *S*-adenosylmethionine as the methyl donor. The anabolic pathway is dependent on the enzyme HGPRT of the purine salvage metabolic pathway, with subsequent multi-enzymatic steps leading to the formation of CYTOTOXIC 6-thioguanine nucleotides. These active metabolites can accumulate in tissues, where they

are catabolized or incorporated into nucleic acids. In addition to its action on 6-mercaptopurine, TPMT can also methylate metabolites of the HGPRT pathway, including thioinosine monophosphate, which is in turn a potent inhibitor of *de novo* purine synthesis. While xanthine oxidase is not believed to play a significant role in 6-mercaptopurine inactivation at the level of hematopoietic tissues, TPMT activity is the principal detoxification pathway for the CYTOTOXIC thioguanine nucleotides in BONE MARROW. Hematopoietic toxicity is thus largely dependent on the activity of TPMT, a POLYMORPHIC ENZYME with genetic variants characterized by low activity and increased risk of severe toxicity in patients [5]. Optimization of AZATHIOPRINE treatment may thus be performed by TPMT genotyping as well as determination of erythrocyte 6-TGN levels.

Clinical indications

Azathioprine is an approved drug for renal transplantation and severe RHEUMATOID ARTHRITIS. Its EFFICACY has also been proven in the management of severe ulcerative colitis, and other autoimmune disorders, including bullous diseases in dermatology [6]. In myasthenia gravis, AZATHIOPRINE, usually in combination with corticosteroids, is a basic immunosuppressive therapy option [7]. Azathioprine is also used as a steroid-sparing agent, and has been administered in cardiac transplantation. The initial dose of AZATHIOPRINE is 3–5 mg/kg/ day; the intravenous formulation may be used postoperatively followed by drug administration by oral route. In combination with CICLOSPORIN and steroids, the dose may be lowered to 1–3 mg/kg/day as a maintenance level. During a 6-month treatment with AZATHIOPRINE along with CICLOSPORIN microemulsion and steroids in recipients of cadaveric kidney transplants, 35% had clinical rejections, compared with 12% over 15 additional months without steroids [2].

Adverse reactions

The dose of AZATHIOPRINE should be reduced in patients administered allopurinol, because the inhibition of xanthine oxidase reduces the metabolic inactivation of AZATHIOPRINE and increases the drug's adverse events. Overall, up to 20% of patients may discontinue treatment due to toxicity [8]. Hematopoietic toxicity, including NEUTROPENIA, anemia and thrombocytopenia, is the most common dose-limiting adverse effect, and it is frequently associated with low TPMT activity. Hepatic toxicity is the second most common adverse event and occurs independently of TPMT activity, which might be correlated with the production of 6-methylmercaptopurine; therefore, therapeutic monitoring of 6-methylmercaptopurine levels may be useful in identifying patients at risk for hepatotoxicity. Gastrointestinal adverse events (mucositis, nausea, vomiting, abdominal pain, diarrhea, pancreatitis), neurotoxicity and photosensitive eruptions may occur. Severe HYPERSENSITIVITY reactions with multiorgan involvement are uncommon. Finally, the incidence of squamous cell carcinoma of the skin and lymphoproliferative malignancies is increased with AZATHIOPRINE [9].

Cyclophosphamide

Introduction

Cyclophosphamide is an antineoplastic and immunosuppressive agent used for the treatment of solid and hematological malignancies as well as severe AUTOIMMUNE DISEASES, including SYSTEMIC LUPUS ERYTHEMATOSUS, sclerodermia, and vasculitis. Although cyclophosphamide has been used in clinical practice since the 1950s, its therapeutic use is still widespread and only partially challenged by the introduction of newer drugs.

Chemical structure

Cyclophosphamide (Fig. 1) is a first-generation oxazaphosphorine ALKYLATING AGENT. It belongs to the group of nitrogen mustards and, like the other members of the family, has the property of becoming strongly electrophilic in body fluids and forming stable, covalent linkages by alkylation of various

nucleophilic moieties, particularly the N7 of guanine residues of DNA.

Mechanism of action and pharmacological effect

Cyclophosphamide acts *via* its principal active metabolite, phosphoramide mustard, through several mechanisms. At the molecular level, phosphoramide mustard is able to bind DNA [10], reacting with purine bases to form double-strand adducts, but at higher doses cyclophosphamide may induce strand nicks by destabilizing purine-sugar bonds with the following loss of purine bases. At the cellular level, the drug is able to trigger APOPTOSIS and to induce a pronounced CYTOTOXIC effect on mature LYMPHOCYTES with relative sparing of the respective precursor cells [11, 12]. The generation of reactive oxygen-free radicals may be considered another mechanism of action, leading to cell death by damaging DNA and inducing lipid peroxidation [13]. Moreover, acrolein – a cyclophosphamide metabolite – seems to be able to inhibit cell proliferation, to induce cell death by APOPTOSIS and to modulate expression of genes and transcription factors, because it reduces the activation of NUCLEAR FACTOR κB (NF-κB) and ACTIVATOR PROTEIN 1 (AP-1). These effects could be increased by the depletion of cellular glutathione, which acts as a detoxifying molecule.

Pharmacokinetics

Cyclophosphamide is well absorbed after oral administration, with a BIOAVAILABILITY greater than 75%. The parent compound is widely distributed throughout the body with low plasma protein binding (20%). The half-life of cyclophosphamide is between 6 and 9 hours, and it is eliminated mainly in the urine as metabolites, even if 5–25% of an intravenous dose is excreted unchanged. Cyclophosphamide is quickly metabolized to active alkylating species by the mixed-function oxidase system of the smooth endoplasmic reticulum of hepatocytes, and maximal concentrations of metabolites in plasma may be observed 2–3 hours after an intravenous dose. Several cytochrome P450 (CYP) isoforms (CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP3A4) are involved in the hydroxylation of the oxazaphosphorine ring of cyclophosphamide [14], leading to 4-OH-cyclophosphamide, which exists in equilibrium with the acyclic tautomer aldophosphamide. Aldophosphamide spontaneously releases acrolein and phosphoramide mustard, the former being a toxic by-product. The involvement of cytochrome P450 in cyclophosphamide metabolism explains why enzyme induction (mainly of CYP2B, CYP3A4, CYP2C8, and CYP2C9 isoforms), which consists of increased cellular RNA and protein contents and associated catalytic activities, occurs following exposure to cyclophosphamide itself. This phenomenon is responsible for increased CLEARANCE and the shortened half-life of the parent drug, because it influences the rate of 4-hydroxylation.

Clinical indications

Cyclophosphamide is used for the treatment of SYSTEMIC LUPUS ERYTHEMATOSUS, vasculitis and other AUTOIMMUNE DISEASES. In MULTIPLE SCLEROSIS, it is most effective in young patients and can be used as induction therapy or as second-line therapy in non-responders to IFN-β [15].

In SYSTEMIC LUPUS ERYTHEMATOSUS, pulse cyclophosphamide at a dose of 1 g/m^2 administered on a monthly schedule ensures a significant advantage in terms of survival and end-stage renal disease with respect to corticosteroids [16]. A remission rate of 75% was observed in patients affected by severe SYSTEMIC LUPUS ERYTHEMATOSUS and treated with high-dose cyclophosphamide (10–15 mg/kg) on a monthly schedule for 6 months, followed by quarterly pulses for 18 months. The same schedule has been adopted in lupus nephritis in children and adults, lowering the relapse rate to less than 10%. Positive results have also been observed in the treatment of optic neuritis associated with SYSTEMIC LUPUS ERYTHEMATOSUS. However, due to toxicity induced by the treatment, it has been proposed that weekly low-dose pulses of 0.5 g are used until disease control is achieved, when it is switched to the monthly schedule and subsequently discontinued [17].

Churg-Strauss syndrome, a granulomatous, necrotizing vasculitis affecting small blood vessels, may be treated with daily doses of cyclophosphamide (2 mg) administered orally in combination with steroids, and the same schedule has been adopted for sclerodermia. Remission of Wegener's granulomatosis, a systemic necrotizing vasculitis affecting small and medium-size vessels, may be obtained with the use of cyclophosphamide, and daily low doses in combination with steroids are effective against the active disease [18]. Cyclophosphamide plus steroids is effective in the treatment of microscopic polyangiitis, a vasculitis that may be associated with severe pulmonary vasculitis and rapidly progressive glomerulonephritis, and polyarteritis nodosa.

It is noteworthy that the EFFICACY or tolerability of cyclophosphamide depends on the amount of phosphoramide mustard within cells, which is controlled by two pathways: (1) aldehyde dehydrogenase, which transforms aldophosphamide to carboxyphosphamide, the major urinary inactive metabolite, and (2) the isozymes CYP2B6 and CYP3A4, which catalyze the dechloroethylation of cyclophosphamide [19]. These mechanisms have been extensively investigated in the experimental and clinical setting. CYP2B6 POLYMORPHISM could be responsible for severe toxicities [20], because this CYP isoform catalyzes the dechloroethylation of cyclophosphamide to the 2- and 3-dechloroethyl metabolite and chloroacetaldehyde, the latter being a toxic by-product. Furthermore, the concentration of aldophosphamide is associated with the expression of aldehyde dehydrogenase isozymes [21]. Thus far, several polymorphic sites have been identified along the gene sequences of CYP and aldehyde dehydrogenase isoforms. Recently, a single nucleotide POLYMORPHISM (SNP) in the ATP-binding cassette, sub-family C4 (ABCC4) efflux pump, a multi-drug resistance (MDR) protein that pumps CYTOTOXIC DRUGS out of cells, was shown to be associated with both adverse gastrointestinal and leukopenic/neutropenic effects of cyclophosphamide [22]. Such pharmacogenetic studies are helping to identify patients at risk of toxicity who should receive a drug other than cyclophosphamide. Finally, O6-alkylguanine-DNA alkyltransferases are enzymes capable of protecting cells from the mutagenic effect of DNA alkylation, and therefore a low expression of these genes in TARGET cells may be associated with a better response to cyclophosphamide therapy.

Adverse reactions

The use of cyclophosphamide is limited by the occurrence of moderate to severe side effects, including gastrointestinal toxicity, alopecia, myelotoxicity, infertility, hemorrhagic cystitis and cardiotoxicity [17, 18]. Nausea and vomiting (10% of treated patients) require adequate prophylactic treatment, with steroids and 5-HT3 antagonists. BONE MARROW toxicity (50–100% of cases) is commonly represented by leukopenia 7–14 days after the drug dose, whereas more severe side effects are agranulocytosis and aplastic anemia. Herpes zoster and other OPPORTUNISTIC INFECTIONS occur in 37% of patients, multiple-organ involvement and lower trough leukocyte counts being additional risk factors of severity of infection. Ovarian failure and decreased sperm counts are associated with infertility (up to 100% of subjects), and their severity correlates with the duration of cyclophosphamide treatment and the patient's age. Adverse events may be mitigated by oral administration of the drug instead of using the intravenous route. The severity of hemorrhagic cystitis may be reduced when the drug is co-administered with the thiol-containing agent mesna, which inactivates acrolein. Because of its MECHANISMS OF ACTION, cyclophosphamide is teratogenic and carcinogenic, the latter effect being more frequent for BONE MARROW (myeloproliferative disorders, 2% of patients) and bladder (transitional cell carcinoma, 2% of patients).

Fludarabine

Introduction

The marked lymphocytolytic activity of fludarabine, a deamination-resistant adenosine analog, in indolent B cell lymphoproliferative disorders has suggested its possible use as an immunosuppressive

agent, although its EFFICACY and potential indications are still a matter of investigation and only limited data are available.

Chemical structure

The synthesis of fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine, F-ara-A, Fig. 1) was achieved by Montgomery and Hewson [23]. Because fludarabine is poorly soluble, the 5'-monophosphate derivative is used for human treatment. Fludarabine 5'-monophosphate is a prodrug that is converted metabolically by dephosphorylation to the active ANTIMETABOLITE moiety by the 5'-nucleotidase activity present in most tissues, including ERYTHROCYTES and endothelial cells.

Mechanism of action and pharmacological effect

Fludarabine enters the cells by the nucleoside transport systems (Fig. 2), mostly through the human equilibrative nucleoside transporter-1 (hENT1) [24]. Fludarabine requires intracellular phosphorylation; the rate-limiting step of drug activation is catalyzed by deoxycytidine kinase (dCK), which phosphorylates fludarabine to F-ara-AMP, whereas 5'-nucleotidase (5'-NT) inactivates the monophosphate metabolite by dephosphorylation [25]. Being a deamination-resistant nucleoside analogue, cytidine deaminase (CdA) has no role in the CLEARANCE of fludarabine from the cells. Fludarabine triphosphate (F-ara-ATP) is the main active metabolite of fludarabine. F-ara-ATP is an alternative SUBSTRATE that competes with the

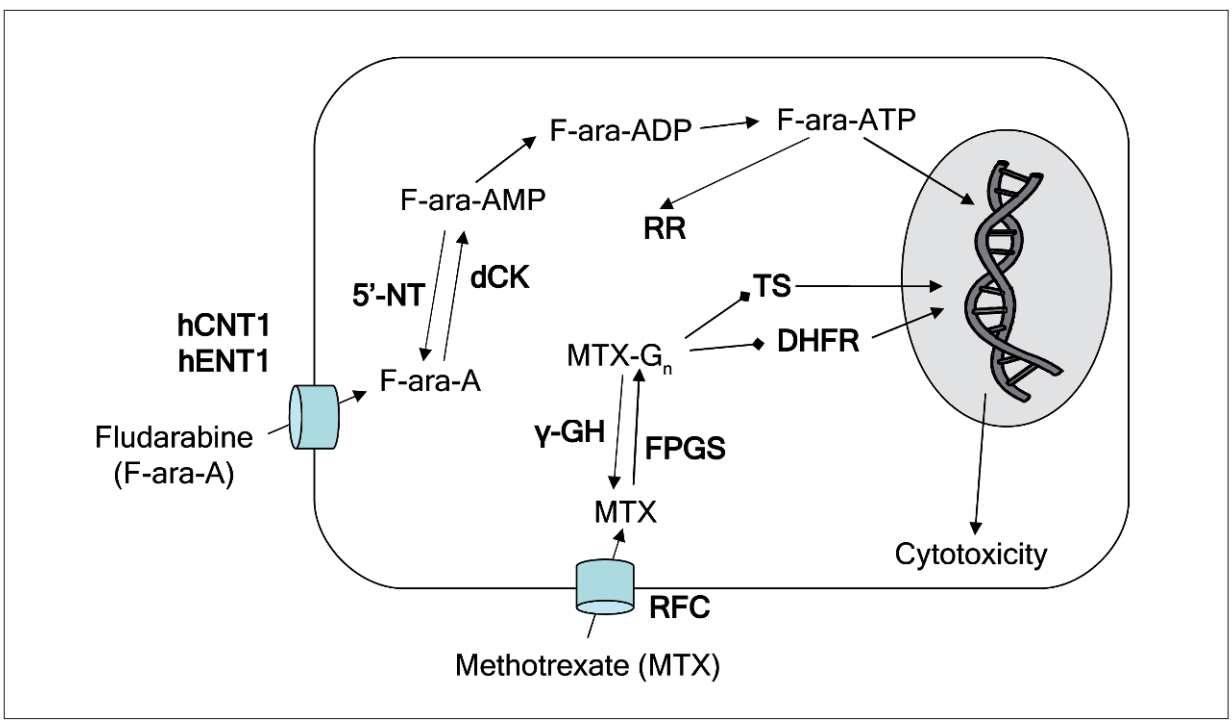

FIGURE 2. MEMBRANE TRANSPORT, METABOLISM AND INTRACELLULAR TARGETS OF FLUDARABINE AND METHOTREXATE
hCNT1, human concentrative nucleoside transporter-1; hENT1, human equilibrative nucleoside transporter-1; dCK, deoxycytidine kinase; 5'-NT, 5'-nucleotidase; CdA, cytidine deaminase; RR, ribonucleotide reductase; RFC, reduced folate carrier; FPGS, folyl-polyglutamate synthase; γ-GH, γ-glutamyl hydrolase; TS, thymidylate synthase; DHFR, dihydrofolate reductase.

normal deoxynucleotide, deoxyadenosine 5'-triphosphate (dATP) and its principal action involves its incorporation into DNA, causing inhibition of RNA and DNA synthesis [25]. F-ara-ATP is an effective inhibitor of ribonucleotide reductase, thus resulting in self-potentiation of its activity by lowering deoxynucleotide pools. Enzymes of DNA synthesis and repair, including DNA polymerases a, b, g and e, DNA primase, DNA ligase I and the nucleotide excision repair system, have been shown to be inhibited by F-ara-ATP [25]. Once incorporated into DNA, F-ara-AMP is a poor SUBSTRATE for subsequent DNA elongation by addition of deoxynucleotides, and behaves as a chain terminator. Inactivation of DNA synthesis is followed by cellular APOPTOSIS by effector CASPASES (i.e., caspase-3). Finally, a unique characteristic of fludarabine is its ability to trigger APOPTOSIS in proliferating as well as in quiescent cells.

Pharmacokinetics

Fludarabine phosphate undergoes extensive first-pass metabolism due to the 5'-nucleotidase activities of ERYTHROCYTES, endothelial cells and parenchymal organs and rapidly disappears from plasma. Peak plasma concentrations (C_{max}) of fludarabine are observed in the first minutes after the end of the infusion; the standard dose of fludarabine (25–30 mg/m^2) results in a C_{max} of about 3 mmol/L. This concentration is adequate to generate intracellular levels of F-ara-ATP capable of triggering the cell death process in lymphoid cells.

Detailed pharmacokinetic data are available with fludarabine administered at doses ranging from 80 to 260 mg/m^2 as a rapid intravenous infusion of 2–5 minutes. Fludarabine displays a tri-exponential decay in plasma, with a $t_{1/2a}$ of about 5 minutes, a $t_{1/2b}$ of 98 minutes and a $t_{1/2g}$ ranging from 6.9 to 19.7 hours. The mean total body CLEARANCE of fludarabine is 4.08 L/h/ m^2, the steady-state volume of distribution is 44.2 L/m^2 and is independent of the drug dose, but is influenced by renal function, thus suggesting the need for dose reduction in patients with impaired kidney function [25].

Pharmacokinetic studies on fludarabine administered at 50, 70 or 90 mg and compared to a corresponding intravenous dose in patients with hematological malignancies, demonstrated that the oral BIOAVAILABILITY of the drug ranges from 55% to about 75% and is independent of the dose [25].

Clinical indications

The use of fludarabine in IMMUNOSUPPRESSION for AUTOIMMUNE DISEASES is still limited. Clinical investigations indicate that the drug may be effective in patients with lupus nephritis [26], PSORIATIC ARTHRITIS [27], chronic immune-mediated demyelinating neuropathies [28], RHEUMATOID ARTHRITIS [29], and refractory dermatomyositis and polymyositis [30]. The schedule of administration is still under investigation. Fludarabine has been given as a single dose of 30 mg/m^2 over 30 minutes, every 4 weeks for four cycles in patients with PSORIATIC ARTHRITIS [27], at 20–30 mg/m^2/day for 3 consecutive days once a month for 6 months [29] or as a single infusion of 25 mg the first month, and then 25 mg for 3 consecutive days each month for 5 months [31] in patients with RHEUMATOID ARTHRITIS. Fludarabine was able to decrease the proliferative response of peripheral LYMPHOCYTES to mitogens, as well as the production of CYTOKINES by T cells (IL-2 and IFN-γ) and MONOCYTES (TNF-α and IL-10) [29]. The successful introduction of the B cell neutralizing anti-CD20 monoclonal ANTIBODY, rituximab, into the therapy of RHEUMATOID ARTHRITIS offers specific immunosuppressive therapy without the side effects of CYTOTOXIC agents, which are now less frequently used in arthritic disorders (see chapter C15).

Adverse reactions

The incidence of drug-related toxicity is dose dependent. While low doses of fludarabine are associated with a good tolerability profile [31], the administration of higher doses may be associated with substantial toxicity [29]. The most common adverse events in patients treated with fludarabine include myelosuppression (NEUTROPENIA, thrombocytopenia and anemia), fever and chills, nausea and vomiting [32]. The duration of clinically significant cytopenia

in a few cases has ranged from 2 months to approximately 1 year. In the treatment of malignant hyperproliferative disorders, fludarabine use is associated with cases of autoimmune hemolytic anemia [33]. Other commonly reported events include malaise, fatigue, anorexia, and weakness. Serious OPPORTUNISTIC INFECTIONS have occurred in patients with chronic lymphocytic leukemia treated with fludarabine.

Methotrexate

Introduction

METHOTREXATE is a folate analog that was introduced into clinical practice more than 50 years ago. It is currently one of the most widely used disease-modifying antirheumatic drugs (DMARDs); its EFFICACY in RHEUMATOID ARTHRITIS has been confirmed in patients refractory or intolerant to other DMARDs or NSAIDs (see chapter C15).

Chemical structure

METHOTREXATE (MTX, amethopterin, *N*-[4-[[(2,4-diamino-6-pteridinyl)methyl]methyl-amino]benzoyl]-L-glutamic acid) (Fig. 1) remains the only antifolate agent used for clinical IMMUNOSUPPRESSION to date. It is a weak dicarboxylic organic acid, and is negatively charged at neutral pH, resulting in limited lipid solubility.

Mechanism of action and pharmacological effect

METHOTREXATE enters cells by the reduced folate CARRIER [34] and its long-chain polyglutamates inhibit the activity of the enzyme dihydrofolate reductase that is involved in fundamental metabolic pathways such as *de novo* synthesis of purines, pyrimidines and polyamines. METHOTREXATE also inhibits thymidylate synthase indirectly by diminishing levels of the enzyme cosubstrate 5,10-methylenetetrahydrofolate, while polyglutamated metabolites of METHOTREXATE also directly bind and inhibit thymidylate synthase (Fig. 2). The use of high-dose METHOTREXATE depletes tumor cells of the purine and pyrimidine precursors required for DNA and RNA synthesis, proliferation and division. Low-dose METHOTREXATE has both immunosuppressive and anti-inflammatory properties, resulting in inhibition of proliferation of CD3 and CD4 LYMPHOCYTES, MONOCYTES/MACROPHAGES and NEUTROPHILS. Indeed, at low concentrations, METHOTREXATE induces APOPTOSIS of activated T cells from human peripheral blood *in vitro*. However, low-dose METHOTREXATE not only acts as a CYTOTOXIC DRUG against immunocompetent cells but also modulates cytokine secretion from T helper LYMPHOCYTES by increasing IL-4 and IL-10 and decreasing IFN-γ and IL-2. Intracellular METHOTREXATE-polyglutamates inhibit the function of 5-amino-imidazole-4-carboxamide ribosyl-5-phosphate formyltransferase (AICAR-formyltransferase). The resulting high concentrations of AICAR lead to enhanced release of adenosine into the blood, activating A2a, A2b and A3 extracellular RECEPTORS on MONOCYTES/MACROPHAGES. In this way, adenosine seems to promote the transcription of mRNA for an IL-1 RECEPTOR antagonist and increases the secretion of the potent anti-inflammatory cytokine IL-10. Moreover, adenosine inhibits the production of TNF-α, IL-6 and IL-8 and the expression of E-SELECTIN on the cell surface [35]. Further details are given in chapter C15.

Pharmacokinetics

After oral administration, active absorption of the drug occurs in the proximal jejunum, which is a saturable process and decreases non-proportionally at increasing oral doses. The extent of absorption is highly variable between patients for doses higher than 10–15 mg/m^2, whereas only a moderate intra-individual pharmacokinetic variability has been described during long-term treatments with low METHOTREXATE doses in patients with psoriasis and RHEUMATOID ARTHRITIS. Low doses of METHOTREXATE are also administered parenterally to ensure compliance and uniform BIOAVAILABILITY. Indeed, the drug is absorbed more rapidly and reaches higher serum concentrations after intramuscular or subcutane-

ous administration compared with the oral route. Methotrexate may also be injected intra-articularly [36].

The volume of distribution of methotrexate corresponds to the intracellular distribution of the drug and, in blood, 30–70% of the drug is bound to albumin. Four hours after oral or intramuscular administration, the concentrations of methotrexate in the synovial fluid are equivalent to plasma concentrations. Methotrexate transport into cells occurs mainly by a carrier-mediated active transport system that methotrexate shares with folates. Once inside the cell, glutamate residues are progressively added to the drug by the folyl-polyglutamate synthetase enzyme. This intracellular accumulation of methotrexate polyglutamates allows a weekly bolus of the drug or the administration of the same dose equally divided in three doses, and relatively high concentrations of the drug are reached in the synovial membrane, cortical and trabecular bone. Methotrexate elimination has been described as biphasic or triphasic with a mean terminal half-life of 6–15 hours. A longer sampling interval is associated with longer terminal half-life estimates of the drug, because of intracellular methotrexate polyglutamylation and the slow release of the drug from cell to plasma.

Methotrexate can be metabolized by three different pathways: (1) in the gastrointestinal tract, intestinal bacteria can degrade the drug to 4-amino-deoxy-N10-methylpteroic acid, a metabolite that usually accounts for less than 5% of the administered dose; (2) in the liver, methotrexate is converted to 7-OH-methotrexate, which is 10-fold less potent at inhibiting dihydrofolate reductase but more nephrotoxic than the parent compound because of its precipitation in acidic urine; despite its extensive binding to serum albumin (>90%), 7-OH-methotrexate does not alter the protein binding of methotrexate; (iii) inside the cells, the drug is converted to pharmacologically active long-chain methotrexate polyglutamates by folyl-polyglutamate synthetase and inactivated by γ-glutamyl hydrolase, potentially contributing to drug resistance [37].

The main elimination route of methotrexate is by renal excretion. The drug is subjected to glomerular filtration and secretion/reabsorption by an active transport system across the renal tubules [38]. In addition, a variable amount of methotrexate is eliminated by active biliary excretion (10–30%), and undergoes enterohepatic recirculation.

Clinical indications

Methotrexate is a first-line systemic agent for the symptomatic control of moderate to severe psoriasis, although its use is being increasingly challenged by newer biologicals [39]. It has become the gold standard, first-line therapy of patients diagnosed with adult rheumatoid arthritis or juvenile arthritis [40,41].

The influence of pharmacogenetics on both the immunosuppressive efficacy and toxicity of methotrexate in rheumatoid arthritis has attracted the interest of researchers. By affecting the intracellular folate pool, the drug influences the activity of the enzyme methylenetetrahydrofolate reductase (MTHFR), an important step in the generation of 5-methyl-tetrahydrofolate, which is the methyl donor for the conversion of homocysteine to methionine. Numerous polymorphisms have been described in the MTHFR gene and among them the C677T polymorphism has been associated with altered phenotypes and higher rates of adverse drug events, at least in oncology studies. The C677T variant of the MTHFR gene leads to alanine to valine substitution and a thermolabile MTHFR with decreased enzyme activity and increased plasma homocysteine levels.

Patients with rheumatoid arthritis receiving methotrexate have been assessed for toxicity, disease activity and the presence of the C677T polymorphism. Patients who are homozygous or heterozygous appear to have an increased risk of methotrexate discontinuation because of adverse events such as gastrointestinal symptoms (e.g., stomatitis, nausea, vomiting), hair loss, rash and hepatotoxicity (increase in transaminases). Thus, the C677T polymorphism seems to make patients with rheumatoid arthritis more sensitive to methotrexate toxicity. However, recent studies challenge this proposed association between the C677T polymorphism and both the efficacy and safety of methotrexate in this indication [42]. SNPs in other enzymes involved in the metabolic pathway of methotrexate, including dihydrofolate reductase and

folyl-polyglutamate synthase, may be better predictors of METHOTREXATE immunosuppressive EFFICACY and toxicity. Hence, further studies are needed to study polymorphisms in other enzymes of the folate pathway and their correlations with drug EFFICACY and toxicity in RHEUMATOID ARTHRITIS.

Adverse reactions

METHOTREXATE has the potential for severe toxicity, particularly to the liver, mostly related to dose or frequency of administration [43]. For this reason, strict monitoring of drug treatment is recommended as most adverse reactions are reversible if detected early. Severe toxicities are managed with leucovorin rescue and hemodialysis with a high-flux dialyzer. Abnormal liver function tests, nausea/vomiting, stomatitis, diarrhea, leukopenia, thrombocytopenia, dermatitis, alopecia and interstitial pneumonitis are adverse reactions observed in patients with RHEUMATOID ARTHRITIS treated with low-dose METHOTREXATE (7.5–15 mg/week). With the exception of a higher incidence of alopecia, photosensitivity, and "burning of skin lesions", the adverse reaction rates in patients suffering from psoriasis are very similar to those with RHEUMATOID ARTHRITIS. In pediatric patients with juvenile arthritis treated with oral, weekly doses of METHOTREXATE (5–20 mg/m^2/week or 0.1–0.65 mg/kg/week), the most common adverse drug reactions are abnormal liver function tests, nausea, vomiting, diarrhea, stomatitis and leukopenia [44].

Mycophenolic acid

Introduction

The development of mycophenolic acid as an immunosuppressive agent was based on the observation that the proliferation of antigen-responsive T and B LYMPHOCYTES preferentially relies on *de novo* purine synthesis, with a negligible contribution of the salvage pathway, which is in turn of primary importance for most cells [45]. The antiproliferative, anticancer activity of the drug is thus of secondary importance with respect to the lymphocytolytic effect, which led to further clinical characterization, particularly for transplant rejection (see chapter C12) and more recently for primary glomerulonephritis.

Chemical structure

Mycophenolic acid (Fig. 1) is a FERMENTATION product of several *Penicillium* species. It is the active moiety released by the prodrugs mycophenolate mofetil, a semisynthetic morpholinoethyl ester of mycophenolic acid, and mycophenolate sodium, which is administered as an enteric-coated formulation designed to prevent upper gastrointestinal tract absorption and reduce the gastrointestinal adverse events seen with mycophenolate mofetil [46].

Mechanism of action and pharmacological effect

Mycophenolic acid is a reversible, non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), and blocks *de novo* purine synthesis in T and B LYMPHOCYTES (Fig. 3), resulting in (1) inhibition of proliferation in response to antigenic stimuli and immunoglobulin production, and (2) initiation of the apoptotic cascade. Two distinct isoforms of IMPDH have been identified, types I and II. IMPDH type I is constitutively expressed mostly in non-replicating cells and IMPDH type II is the inducible, predominant enzyme in activated LYMPHOCYTES. IMPDH type II is approximately five times more susceptible to inhibition by mycophenolic acid than type I, and this difference explains the unique susceptibility of proliferating LYMPHOCYTES to depletion of purine bases by mycophenolic acid. In contrast to CALCINEURIN INHIBITORS, mycophenolic acid has no effect on the production or release of CYTOKINES [47]. Additional pharmacological effects include suppression of ANTIBODY production, as a consequence of failure of B cell activation, suppression of dendritic cell maturation, with a resulting decrease in antigen presentation to T LYMPHOCYTES, and reduced recruitment of MONOCYTES into sites of graft rejection and INFLAMMATION [48]. Anti-inflammatory effects may

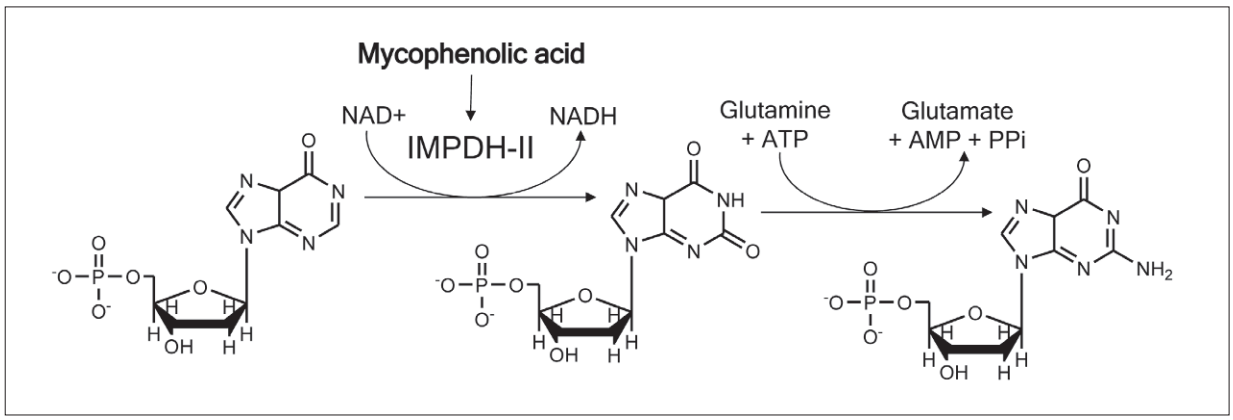

Figure 3. Inhibition of inosine monophosphate dehydrogenase (IMPDH) type II by mycophenolic acid
PPi, pyrophosphate (inorganic)

also result from decreased expression and activity of the inducible form of nitric oxide synthase [49].

Pharmacokinetics

Mycophenolate mofetil (Fig. 4) is absorbed in the stomach and the ester linkage is rapidly hydrolyzed by ubiquitous esterases to yield mycophenolic acid, the active immunosuppressive moiety [50]. Therefore, mycophenolate mofetil is undetectable in the circulation, even after intravenous administration. In contrast, enteric-coated mycophenolate sodium (Fig. 4) is mainly absorbed in the small intestine. The oral bioavailability of mycophenolic acid from mycophenolate mofetil and mycophenolate sodium is 94% and 71%, respectively, and the peak plasma concentration (C_{max}) occurs 1–2 hours after oral administration [46, 51]. After a dose of 1 g mycophenolate mofetil, the maximum plasma concentration ranges from 10 to 30 mg/L in patients with stable renal function, and the elimination half-life averages 17 hours, while pre-dose levels are approximately 1 mg/L [52]. The area under the curve (AUC) of mycophenolic acid is the most significant pharmacokinetic parameter for therapeutic drug monitoring; indeed, low mycophenolic acid AUC is significantly associated with an increased risk of acute rejection of kidney graft [50]. In patients given doses of mycophenolate mofetil tailored to achieve low (16.1), intermediate (32.2) or high (60.6 mg/h/L) total AUC of mycophenolic acid, a highly significant relationship was found between the AUC of the active metabolite and the incidence of rejection. In the low, intermediate and high AUC groups the incidence was 27.5%, 14.9% and 11.5%, respectively [53]. There was also a higher rate of premature withdrawal from the study as the AUC of mycophenolic acid increased: 7.8%, 23.4% and 44.2% for the three groups, respectively [53]. Pharmacokinetic monitoring can be performed on the basis of $AUC_{0–2h}$ of mycophenolic acid, this parameter being in good agreement with the $AUC_{0–12h}$ measured across the complete dosing interval period.

Mycophenolic acid undergoes extensive hepatic glucuronidation by glucuronosyl-transferases (UGT) isoforms 1A8, 1A9 and 1A10 [54] to form the inactive metabolite mycophenolic acid glucuronide, which is excreted into the bile (Fig. 4). The glucuronyl moiety of the metabolite is cleaved by enteric β-glucuronidases of intestinal bacteria to release mycophenolic acid, which undergoes entero-hepatic recirculation producing a secondary peak plasma concentration about 6–12 h after administration. More than 90% of a dose of mycophenolate mofetil is excreted in the urine as mycophenolic acid glucuronide [55]. The comparison of pharmacokinetic parameters of mycophenolate sodium 720 mg and mycophenolate mofetil 1000 mg revealed that

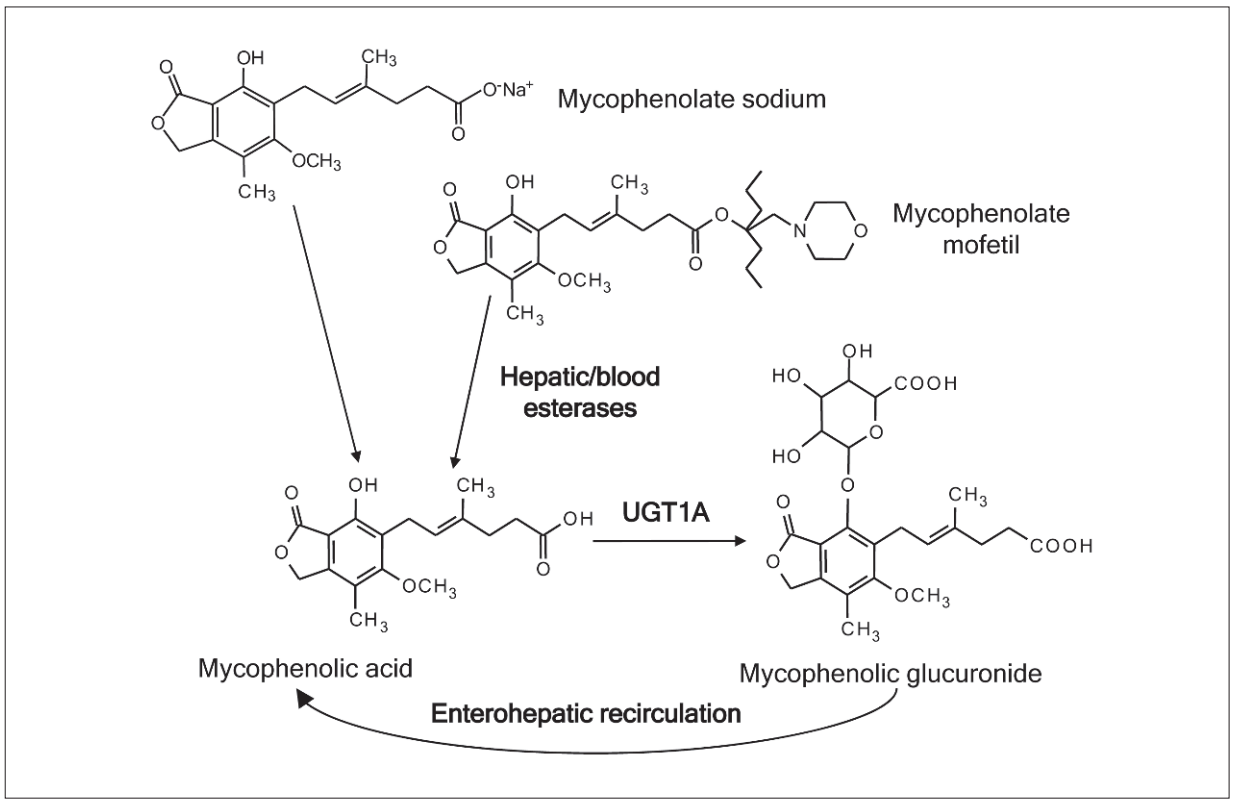

FIGURE 4.

Metabolism of mycophenolate mofetil and mycophenolate sodium to the active moiety mycophenolic acid; UGT1-dependent metabolism yields mycophenolate glucuronide, which undergoes enterohepatic recirculation.

C_{max} and $AUC_{0–24h}$ in the former group were consistently higher than in the latter group. Overall, there was a mean increase of 32% in systemic mycophenolic acid exposure over the length of the study in the mycophenolate sodium-treated patients [46].

Clinical indications

Mycophenolate mofetil is administered at 1–3 g/day orally or, less frequently, by the intravenous route, and it is indicated for the prophylaxis of organ rejection in patients receiving allogeneic renal, cardiac or hepatic transplants mainly in combination with a CALCINEURIN INHIBITOR (TACROLIMUS or CICLOSPORIN) and corticosteroids. Clinical studies indicate that mycophenolate mofetil, administered in combination with immunosuppressive ANTIBODIES, can be used in calcineurin- and steroid-sparing regimens, to reduce the toxicity burden of IMMUNOSUPPRESSION [56]. Mycophenolate sodium 720 mg twice daily has been compared with mycophenolate mofetil 1000 mg twice daily with respect to safety and EFFICACY. Both formulations demonstrated equivalent EFFICACY and safety in renal transplant recipients; in particular, the incidence of gastrointestinal adverse events was similar between the formulations despite the higher serum levels achieved with mycophenolate sodium [46]. Based on its antifibrotic and antiproteinuric effects, mycophenolate mofetil is now being used increasingly in patients with primary glomerulonephritis, particularly those with idiopath-

ic nephritic syndrome or drug-induced deterioration of renal function [57].

Clinically important drug interactions of mycophenolate mofetil and mycophenolate sodium involve acyclovir/valacyclovir (increased hematological toxicity), cholestyramine, antacids containing aluminum hydroxide/magnesium hydroxide, CICLOSPORIN (reduced BIOAVAILABILITY of mycophenolic acid), and levonorgestrel (reduced AUC of the hormone) [46].

Adverse reactions

Overall, mycophenolate mofetil is better tolerated than AZATHIOPRINE. The drug produces a lower incidence of leukopenia and fewer immunosuppressive-related malignancies compared with AZATHIOPRINE and lacks the neurotoxicity and nephrotoxicity associated with CALCINEURIN INHIBITORS. The most commonly observed adverse events associated with mycophenolate mofetil and mycophenolate sodium are gastrointestinal (i.e., nausea, vomiting, diarrhea, constipation, dyspepsia, flatulence, anorexia), hematological (leukopenia, thrombocytopenia, anemia), and OPPORTUNISTIC INFECTIONS. Patients experiencing gastrointestinal or hematological adverse events usually respond to dose fractionation or reduction. However, frequent dose changes have been associated with poorer outcomes, including a higher incidence of graft loss. A recent review of comparative studies of mycophenolate mofetil and AZATHIOPRINE in liver transplantation indicates that mycophenolate mofetil is not necessarily superior to the older agent, and proposes further investigation of these immunosuppressive therapies [2].

Conclusions

The increasing understanding of the pathophysiology of AUTOIMMUNE DISEASE and graft rejection has revealed a number of potential targets that have been exploited for the design of potent immunosuppressive drugs, including MONOCLONAL ANTIBODIES and calcineurin-binding agents.

However, the occurrence of multiorgan toxicity associated with the use of CALCINEURIN INHIBITORS, the severe metabolic adverse events induced by corticosteroids, the potential for long-term cancer risk associated with the use of selected MONOCLONAL ANTIBODIES and the occurrence of infectious diseases due to over-IMMUNOSUPPRESSION soon became key management issues that urged a re-evaluation of immunosuppressive treatment schedules. In this context, CYTOTOXIC DRUGS, particularly ANTIMETABOLITES, still play a crucial role in the control of IMMUNE SYSTEM activation, particularly in steroid- and calcineurin-inhibitor-resistant diseases, and are expected to be instrumental in the long-term IMMUNOSUPPRESSION maintenance in steroid- and calcineurin-binding-sparing schedules.

Summary

CYTOTOXIC immunosuppressive drugs are a group of HETEROGENEOUS compounds characterized by their ability to damage immune cells by nonspecific mechanisms, including nucleotide pool depletion, incorporation into DNA and nucleic acid alkylation. Their successful clinical use in IMMUNOSUPPRESSION for organ transplantation and AUTOIMMUNE DISEASES has been proven in a large number of clinical trials.

Azathioprine is an ANTIMETABOLITE prodrug of 6-mercaptopurine with an imidazolyl group attached to the SH group of 6-mercaptopurine. Active metabolites of AZATHIOPRINE are inhibitors of nucleic acid synthesis through impairment of the *de novo* purine biosynthetic pathway and incorporation into RNA and DNA. The drug suppresses the proliferation of T and B LYMPHOCYTES and reduces the number of CYTOTOXIC T cells and plasma cells in circulation and peripheral organs. Azathioprine is mainly metabolized by xanthine oxidase; however, thiopurine methyltransferase (TPMT) is the principal detoxification pathway for the CYTOTOXIC thioguanine nucleotides in BONE MARROW. Hematopoietic toxicity is dependent, at least in part, on the activity of TPMT, a POLYMORPHIC ENZYME with genetic variants characterized by low activity and increased risk of severe toxicity in patients. Azathioprine is used in renal transplan-

tation, RHEUMATOID ARTHRITIS ulcerative colitis, and other skin autoimmune disorders. Hematopoietic toxicity, including NEUTROPENIA, anemia and thrombocytopenia, and hepatic toxicity are the most common adverse events and may be predicted by TPMT genotyping.

Cyclophosphamide is a first-generation oxazaphosphorine ALKYLATING AGENT belonging to the group of nitrogen mustards, characterized by the ability to form stable, covalent linkages by alkylation of the N7 of guanine residues of DNA *via* its active metabolite phosphoramide mustard. At the cellular level, the drug is able to trigger APOPTOSIS and to induce a pronounced CYTOTOXIC effect on mature LYMPHOCYTES. Cyclophosphamide has a good oral BIOAVAILABILITY, and it is eliminated mainly in the urine as metabolites. Cyclophosphamide is transformed to active alkylating species by the mixed-function oxidase system of the smooth endoplasmic reticulum of hepatocytes. Several cytochrome P450 (CYP) isoforms (CYP2A6, CYP2B6, CYP2C8, CYP2C9, and CYP3A4) are involved in the hydroxylation of the oxazaphosphorine ring of cyclophosphamide, leading to 4-OH-cyclophosphamide, which exists in equilibrium with aldophosphamide. Aldophosphamide spontaneously releases acrolein and phosphoramide mustard, the former being a toxic by-product. The EFFICACY or tolerability of cyclophosphamide depends on the amount of phosphoramide mustard within cells. In this respect, key enzymes are aldehyde dehydrogenase, which transforms aldophosphamide to carboxyphosphamide, the major urinary inactive metabolite, and the isozymes CYP2B6 and CYP3A4, which catalyze the dechloroethylation of cyclophosphamide to 2- and 3-dechloroethyl-metabolite and chloroacetaldehyde, the latter being a neurotoxic by-product. The drug is used for the treatment of SYSTEMIC LUPUS ERYTHEMATOSUS, vasculitis and other AUTOIMMUNE DISEASES. The use of cyclophosphamide is limited by the occurrence of moderate to severe side effects, including gastrointestinal toxicity, alopecia, myelotoxicity, infertility, hemorrhagic cystitis and cardiotoxicity.

Fludarabine, a deamination-resistant adenosine analogue, with marked lymphocytolytic effect, is a prodrug that requires intracellular phosphorylation. The rate-limiting step of drug activation is catalyzed by deoxycytidine kinase (dCK), which phosphorylates fludarabine to F-ara-AMP. Fludarabine triphosphate (F-ara-ATP) is the main active metabolite of fludarabine. F-ara-ATP is an alternative SUBSTRATE that competes with the normal deoxynucleotide, deoxyadenosine 5'-triphosphate (dATP), and it is incorporated into DNA, causing inhibition of RNA and DNA synthesis. F-ara-ATP is an effective inhibitor of ribonucleotide reductase, thus resulting in self-potentiation of its activity by lowering deoxynucleotide pools. Inactivation of DNA synthesis is followed by cellular APOPTOSIS by effector CASPASES. A unique characteristic of fludarabine is its ability to trigger APOPTOSIS in proliferating as well as in quiescent cells. Fludarabine is administered by intravenous and oral routes as a phosphate prodrug and undergoes extensive metabolism due to the 5'-nucleotidase activities of ERYTHROCYTES, endothelial cells and parenchymal organs, and rapidly disappears from plasma to release fludarabine. Fludarabine displays a tri-exponential decay in plasma, and drug CLEARANCE is influenced by renal function. The use of fludarabine in IMMUNOSUPPRESSION for AUTOIMMUNE DISEASES is still limited and challenged by the increasing use of the biological, rituximab. Preliminary clinical data indicated that fludarabine may be active in patients with lupus nephritis, PSORIATIC ARTHRITIS, inflammatory neuropathies, RHEUMATOID ARTHRITIS, and refractory dermatomyositis and polymyositis. The incidence of drug-related toxicity is dose dependent; the most common adverse events being myelosuppression, fever, nausea and vomiting. Cases of autoimmune hemolytic anemia have also been reported. Other commonly reported events include malaise, fatigue, anorexia, and weakness. Serious OPPORTUNISTIC INFECTIONS have occurred in patients with chronic lymphocytic leukemia treated with fludarabine.

METHOTREXATE, a folate analogue, is currently the gold standard, first-line disease-modifying antirheumatic drug (DMARDs) in adult RHEUMATOID ARTHRITIS and juvenile arthritis. The drug enters cells by the reduced folate CARRIER and its long-chain polyglutamates inhibit the enzyme dihydrofolate reductase, thereby blocking *de novo* synthesis of purines and pyrimidines; METHOTREXATE also inhibits thymidylate synthase. Low-dose METHOTREXATE has both immunosuppressive and anti-inflammatory properties result-

ing in inhibition of proliferation of CD3 and CD4 LYMPHOCYTES, MONOCYTES/MACROPHAGES and NEUTROPHILS. Oral BIOAVAILABILITY of METHOTREXATE is predictable after administration of low doses, while higher doses should be administered parenterally. The main elimination route of METHOTREXATE is by renal excretion. In addition, a variable amount is eliminated by active biliary excretion and undergoes enterohepatic recirculation. METHOTREXATE is indicated for the management of RHEUMATOID ARTHRITIS, juvenile arthritis and psoriasis. The drug has the potential for severe toxicity, including hepatotoxicity, nausea/vomiting, stomatitis, diarrhea, leukopenia, thrombocytopenia, dermatitis, alopecia and interstitial pneumonitis. The homozygous or heterozygous condition of C677T POLYMORPHISM of the enzyme methylenetetrahydrofolate reductase (MTHFR) may be associated with an increased risk of adverse events, but findings are inconclusive.

Finally, mycophenolic acid is an immunosuppressive agent that exerts a reversible, non-competitive inhibition of inosine monophosphate dehydrogenase (IMPDH), and blocks *de novo* purine synthesis in T and B LYMPHOCYTES. At variance with CALCINEURIN INHIBITORS, mycophenolic acid has no effect on the production or release of CYTOKINES, but inhibits antigen-presentation by DENDRITIC CELLS to T cells. Additional pharmacological effects include suppression of ANTIBODY production, as a consequence of failure of B cell activation, as well as anti-inflammatory effects resulting from decreased expression and activity of the inducible NITRIC OXIDE SYNTHASE. The oral BIOAVAILABILITY of mycophenolic acid from mycophenolate mofetil and mycophenolate sodium is high, and the drug undergoes extensive hepatic glucuronidation by glucuronosyl-transferases (UGT) isoforms 1A8, 1A9 and 1A10 to form the inactive metabolite mycophenolic acid glucuronide, which is excreted into the bile. The drug is indicated for the prophylaxis of organ rejection in patients receiving allogeneic renal, cardiac or hepatic transplants mainly in combination with a CALCINEURIN INHIBITOR and corticosteroids. Overall, mycophenolate mofetil is better tolerated than AZATHIOPRINE. The drug produces a lower incidence of leukopenia and fewer IMMUNOSUPPRESSION-related malignancies compared with AZATHIOPRINE and lacks the neurotoxicity and nephrotoxicity associated with CALCINEURIN INHIBITORS. The most commonly observed adverse events associated with mycophenolate mofetil and mycophenolate sodium are gastrointestinal (i.e., nausea, vomiting, diarrhea, constipation, dyspepsia, flatulence, anorexia) and hematological (leukopenia, thrombocytopenia, anemia), as well as OPPORTUNISTIC INFECTIONS.

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Immunosuppressives in transplant rejection

C12

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Introduction

Suppression of immune reactivity can either be an undesirable effect or a situation that is specifically induced to the benefit of a patient. Examples of the first come from immunotoxicology, e.g., XENOBIOTICS or environmental factors causing IMMUNOSUPPRESSION. Virus infections, as exemplified by HUMAN IMMUNODEFICIENCY VIRUS (HIV), can cause severe immunodeficiency. Under clinical conditions suppression of the IMMUNE SYSTEM is specially indicated in two indications: AUTOIMMUNITY and organ transplantation. In the conventional approach, AUTOIMMUNE DISEASES like RHEUMATOID ARTHRITIS (RA) are mainly treated by inhibition of the effector phase with anti-inflammatory drugs like corticosteroids (see chapter C13) and ANTIMETABOLITES like METHOTREXATE (MTX) (see chapter C11). During the last decade new treatments have been introduced, including BIOLOGICALS [MONOCLONAL ANTIBODIES (mAb) and fusion proteins] with anti-inflammatory activity. In addition, immunosuppressants like CICLOSPORIN (CsA), which were at first developed for transplantation, are increasingly used in AUTOIMMUNE DISEASES, and some like LEFLUNOMIDE (see below) have been developed for RA as the first indication.

In contrast, in organ transplantation, there is a principal need for interference with the initiation of an immune response that is induced by the grafted organ. Generally, high-dose IMMUNOSUPPRESSION is needed in the first period after transplantation (INDUCTION TREATMENT), or in the treatment of rejection episodes [1, 2]. To keep graft function stable, so-called MAINTENANCE TREATMENT is given. Originally, when transplantation was introduced as a treatment of end stage organ failure (the first kidney transplant was performed in the early fifties of the last century), there were few possibilities to prevent or treat ALLOGRAFT rejection. In the sixties and early seventies, this was mainly restricted to combinations of AZATHIOPRINE (AZA), corticosteroids, and cyclophosphamide (CY), see also chapter C11. Combinations of these drugs were effective, but associated with severe side effects, mainly related to BONE MARROW depression (myelosuppression, leukopenia, anemia) and gastrointestinal symptoms. A more specific reagent, anti-lymphocyte globulin (ALG), became available in 1966 and was used in INDUCTION TREATMENT immediately after transplantation. Based on the complications when working with these drugs, kidney transplantation developed slowly, and heart transplantation did not develop after it was first performed in 1963 because of a lack of efficacious immunosuppressive regimens. The history of transplantation during the second half of the 20th century has witnessed a major search for innovative approaches to suppress or modulate the immune response toward a graft [3].

The most widely used immunosuppressives at present are XENOBIOTICS, i.e., orally active drugs produced by micro-organisms or chemically synthesized molecules (structural formulas of some examples are given in Fig. 1). A landmark in IMMUNOSUPPRESSION for transplantation was the introduction of CsA, an inhibitor of intracellular calcineurin, in 1983. Using CsA as a BASELINE IMMUNOSUPPRESSANT in combination with AZA and corticosteroids, 1-year graft survival in kidney transplantation increased to 80–90% and also heart transplantation reached a 1-year patient survival exceeding 80%.

Currently, INDUCTION IMMUNOSUPPRESSIVE TREATMENT, during the first 2–4 weeks after transplantation, includes either triple therapy, i.e., a CALCINEURIN INHIBITOR, an inhibitor of cell proliferation like AZA or MPA, and corticosteroids as an anti-inflammatory agent; or quadruple therapy in which an Ab is added

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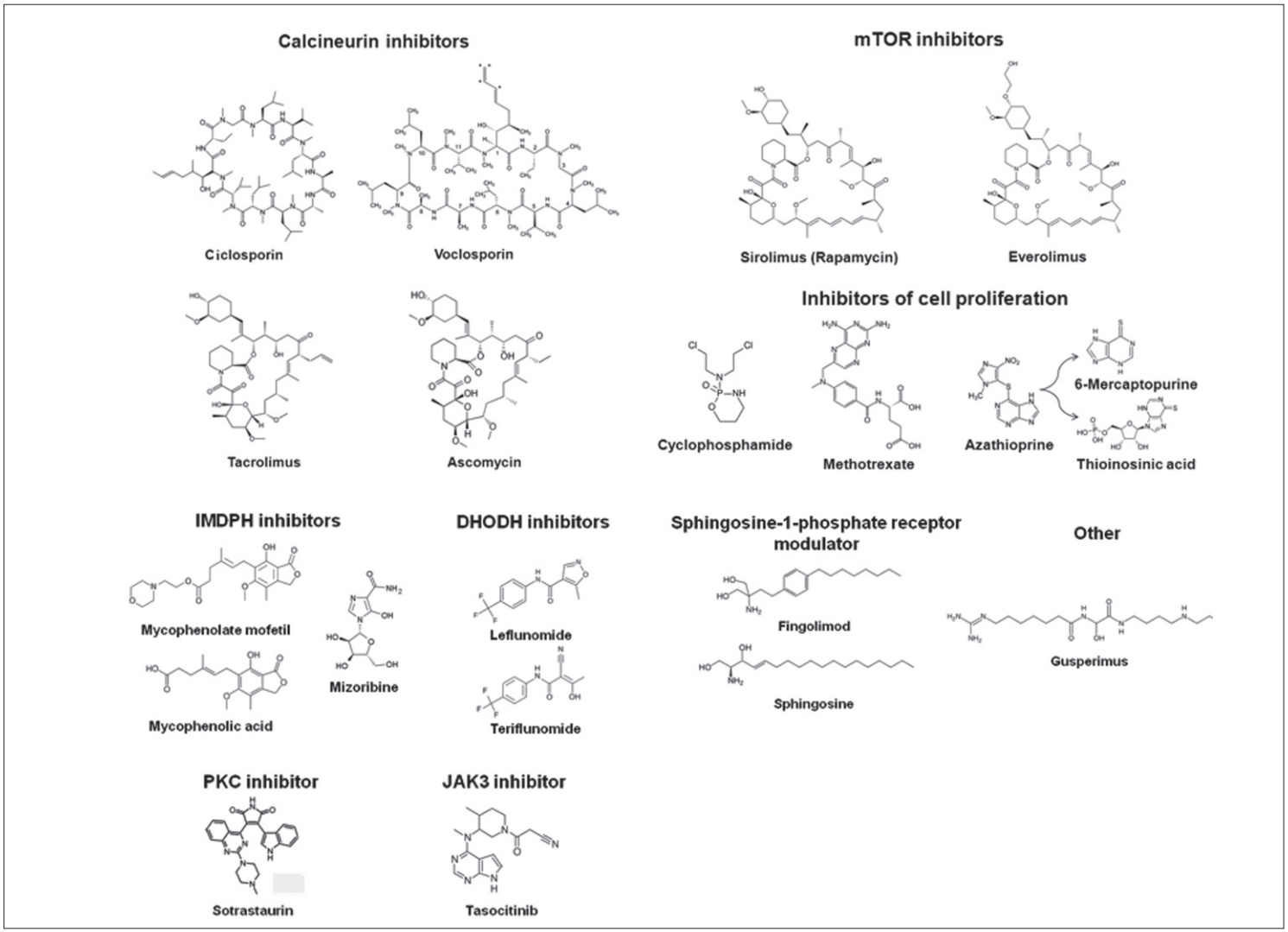

Figure 1

Structures of xenobiotic immunosuppressives: calcineurin inhibitors, inhibitors of the mammalian target of rapamycin (mTOR), cell proliferation, inosine monophosphate dehydrogenase (IMPDH), dihydroorotate dehydrogenase (DHODH), protein kinase C (PKC) and Janus kinase 3 (JAK3), sphingosine-1-phosphate receptor modulator, and gusperimus.

to this regimen. Examples are a polyclonal Ab (pAb), anti-thymocyte globulin (ATG) or ALG, or a more specific mAb such as anti-CD3 Ab directed to the T CELL RECEPTOR (TCR) on T LYMPHOCYTES, or anti-CD25 Ab directed to the IL-2 RECEPTOR on activated T LYMPHOCYTES. Many alternatives are currently possible in combination drug treatment, in which one of the drugs is replaced by another, such as an inhibitor of the mammalian TARGET of rapamycin (mTOR), which inhibits growth factor-induced cell proliferation.

Most drugs, in particular those with a narrow therapeutic window between EFFICACY and side effects, require regular monitoring of exposure on the basis of blood concentrations. For others, hematological parameters serve as a SURROGATE marker (like blood leukocyte counts in the case of AZA). Side effects of CALCINEURIN INHIBITORS primarily concern the kidney, and therefore in a number of kidney transplant centers patients are given such drugs only after good kidney function is achieved. When patients show stable graft function, the INDUCTION TREATMENT is gradually converted into MAINTENANCE TREATMENT, in which lower doses are used, or one component is gradually tapered down and eliminated from the regimen. Corticosteroids are an example of this approach, because of the endocrinological side effects (most visible are the Cushingoid features, but others include OSTEOPOROSIS, diabetes, hyperlipidemia, hypertension, hirsutism, and cataracts). In many transplant centers steroid-sparing regimens are the first goal in MAINTENANCE TREATMENT. Rejection crises (documented by histopathology of a graft biopsy, or biochemical markers in blood) are normally treated first with high-dose intravenous corticosteroids (bolus injections on 3–5 successive days); so-called steroid-resistant rejections can be treated by Ab, either anti-CD3 Ab, ATG or in severe forms, ALG.

BIOLOGICALS (i.e., pAb or mAb and fusion proteins generated by rDNA technology) have long been considered as a new type of promising innovative immunosuppressants. This started with the introduction of the anti-lymphocyte pAb ALG and the mouse anti-CD3 mAb muronomab in the sixties and eighties of the last century, respectively, and was followed by the introduction of HUMANIZED or CHIMERIC anti-IL-2 RECEPTOR Ab for the primary indication of transplantation. The main advantage of BIOLOGICALS is their higher SPECIFICITY, resulting in a broadened therapeutic window. The main disadvantage is their administration route (parenteral instead of oral for most low-molecular weight XENOBIOTICS) and their potential immunogenicity, e.g., the formation of anti-mouse Ab in the case of a mouse reagent. In contrast to low molecular weight XENOBIOTICS, most BIOLOGICALS work extracellularly, i.e., at TARGET cell surface molecules. Their potential side effects are therefore mediated by cross-reactivity with other cell populations than the primary TARGET cell. In general, therapeutic Ab to cell surface molecules can affect the TARGET cell by two mechanisms: either by temporary blockade or down-regulation of surface molecules, resulting in dysfunction or anergy, or by lysis of the cell, e.g., by the induction of APOPTOSIS, complement-mediated lysis or by ANTIBODY-dependent cellular CYTOTOXICITY (ADCC, depleting Ab).

The field of BIOLOGICALS is progressing and a number of new reagents have been launched during the last decade or are in advanced development. To avoid the formation of anti-mouse Ab, mAb for clinical application are currently generated by genetic engineering. Three major approaches are followed: (1) CHIMERIC Ab, in which the constant part of Ig heavy and light chains in the mouse Ab molecule is replaced by human Ig sequences; (2) HUMANIZED Ab, in which the sequence encoding the complementarity DETERMINING REGION (CDR) of the variable part of the mouse Ab is inserted into the sequence encoding human Ig; and (3) fully human Ab in which the Ab is produced by a human cell line and not a mouse cell line after immunization. The second approach may lead to a loss of binding AFFINITY, as has been shown for the HUMANIZED anti-CD25 Ab DACLIZUMAB (mentioned below). Murine antigenic determinants are still present in the variable part of light and heavy chains, albeit less so in HUMANIZED Ab, and hence the molecules are still immunogenic, although the immunogenicity is strongly reduced. To reduce this further, current technology allows for the generation of completely human anti-human Ab. The selection of a relevant Ab is performed using the product of cell lines created by the relevant Ab-encoding sequences inserted into a high-performance human Ab-producing cell line. Engineered Ab have the

advantage that the $t_{½}$ in the circulation can be substantially longer, e.g., 24–48 hours for a mouse Ab to 2–4 weeks for an engineered human(ized) Ab.

During the last decade many new immunosuppressants have been introduced to the market or have entered advanced clinical development, both XENOBIOTICS and BIOLOGICALS. The main targets in transplantation are solid organs, including kidney, heart, liver and lung, which will be the main focus of this chapter. Structural formulas for major XENOBIOTICS are presented in Figure 1, and a summary of immunosuppressants is given in Table 1 (see pp 530–533). A selection of general overview papers is given in the selected readings list.

The immunosuppressive armamentarium clearly enables the clinician to use a variety of reagents that TARGET various parts of the immune response, i.e., depletion of specific lymphocyte subpopulations, cell trafficking and adhesion, costimulatory blockade in antigen presentation, TCR signaling, B lymphocyte signaling, cytokine signaling, and cell proliferation. This is illustrated in Figure 2 for a number of drugs targeting cell surface molecules and intracellular pathways, and in Figure 3 for cell surface molecules with a role in antigen presentation and COSTIMULATION. Nearly all xenobiotic drugs work intracellularly, by inhibition of early or late events in intracellular signaling after lymphocyte activation, or by inhibition of cell proliferation (direct interference in DNA/RNA synthesis) following activation. As these intracellular pathways are not truly selective for LYMPHOCYTES, most drugs manifest a quite narrow therapeutic window. Therefore, combined used of drugs is common practice to optimally exploit synergy in IMMUNOSUPPRESSION, while avoiding adverse side effects. Besides immunosuppressive activity, a number of reagents, in particular those targeting CYTOKINES, manifest anti-inflammatory activity. For a number of reagents, i.e., those targeting COSTIMULATION in antigen presentation, IMMUNOMODULATION ('TOLERANCE' induction) has been claimed. CALCINEURIN INHIBITORS such as CsA and TACROLIMUS are widely used in transplant recipients together with AZA, but in a number of centers have been replaced by MPA derivatives, MTOR INHIBITORS, and blockers of COSTIMULATION. Remarkably, most xenobiotic immunosuppressives originated from anti-infection or cancer drug development programs; because of their failure in these indications or because IMMUNOSUPPRESSION was observed as a 'side effect', the drugs were subsequently developed as immunosuppressants. One exception is TACROLIMUS, which was specifically developed as an immunosuppressant and turned out to have the same mechanism of action as CsA. Other exceptions are the protein kinase C (PKC) inhibitor sotastaurin and the Janus-activated kinase 3 (JAK3) inhibitor tasocitinib, which are in advanced clinical development, and originated from a program on IMMUNOSUPPRESSION. Some new immunosuppressants were first developed for other indications, used off-label in transplant patients and subsequently evaluated in clinical trials in transplantation. In general, dose levels in these non-transplant indications are lower than those used in the transplantation setting.

With the present spectrum of immunosuppressants and extensive clinical experience with various drug combinations, the management of IMMUNOSUPPRESSION in patients with transplanted organs has markedly improved. However, major complications that are directly associated with IMMUNOSUPPRESSION still occur, such as drug toxicity, increased susceptibility to infection, and development of tumors, in particular so-called post-transplant lymphoproliferative disease [4–6]. On the other hand, long-term graft survival is hampered by graft dysfunction due to CHRONIC REJECTION. This phenomenon not only relates to the ongoing (smouldering) immune response of the recipient to the graft, but also to intrinsic changes in the graft itself, mainly regarding the vasculature, so-called graft vessel disease or accelerated graft arteriosclerosis. This involves thickening of the intima of blood vessels of the graft and is ascribed to migration of smooth muscle cells to this site followed by cellular proliferation and extracellular matrix formation. In lung transplantation, CHRONIC REJECTION becomes manifest as an obstruction called bronchiolitis obliterans, in which the bronchioles are compressed and narrowed by fibrosis or INFLAMMATION. With prolonged graft survival, CHRONIC REJECTION is now a major cause of graft loss in long-term surviving patients [7, 8]. There are no specific drugs available for the prevention or treatment of CHRONIC REJECTION: some anti-inflammatory drugs, like azithromycin in

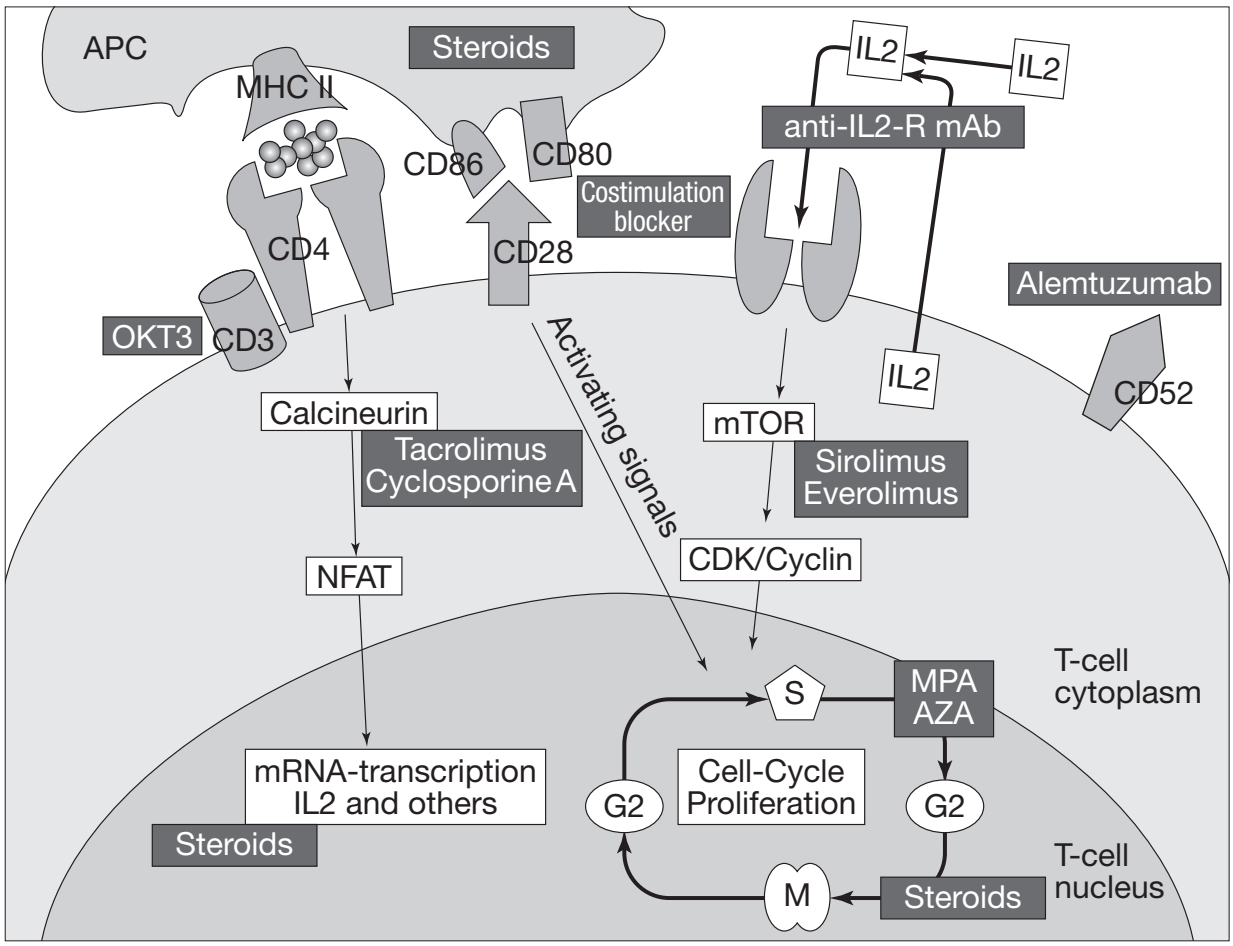

Figure 2. Mechanisms of action of various immunosuppressive agents in T lymphocytes

Alemtuzumab and anti-CD3 mAb (OKT3) target specific lymphocytic surface structures to induce cytolysis or receptor down-regulation; a similar principle is involved with anti-lymphocyte and anti-thymocyte globulins (not illustrated) which target multiple molecules on the cell surface. Signal transduction from the TCR/CD3 complex (so-called signal 1) is blocked by calcineurin inhibitors (tacrolimus and CsA), which affect nuclear factor of activated T cells (NFAT). Costimulatory signaling (signal 2) via the interaction between CD28 and B7 (CD80, CD86) is blocked by the fusion proteins abatacept and belatacept. Not shown is the costimulatory pathway via the CD40-CD154 interaction (see Fig. 3). Protein kinase C is involved in processes downstream of signal 1 and 2, and this step in intracellular signal transduction is inhibited by sotrastaurin (not illustrated). Anti-CD25 mAb basiliximab and daclizumab (anti-IL-2-R mAb) target the receptor for IL-2, required for cell activation mediated by cytokines (signal 3). This pathway involves the mammalian target of rapamycin (mTOR), which is targeted by sirolimus and everolimus and the cyclin-dependent kinase (CDK), and the JAK/STAT pathway, which is targeted by tasocitinab (not shown). Mycophenolic acid (MPA) derivatives and azathioprine (AZA) interfere with the cell cycle, preventing T (and B) lymphocyte proliferation. Steroids target multiple sites in the process. Modified from Urschel et al. [selected reading].

Table 1. Lymphocyte-targeting pharmaceuticals currently approved or in advanced stage of development

Reagent[1]	Trade name	Mechanism of action	Status[2]
Cell proliferation, antimetabolites			
Cyclophosphamide (CY)	Endoxan, Cytoxan, Neosar, Procytox, Revimmune		
Azathioprine (AZA)	Azasan, Imuran	Purine synthesis inhibitor; prodrug of 6-mercaptopurine, 6-thioinosinic acid	Transplantation, autoimmune disease
Mizoribine	Bredinin	Inhibitor of IMPDH	Transplantation, RA, autoimmune disease
Mycophenolic mofetil	Cellcept	Inhibitor of IMPDH	Transplantation
Mycophenolic sodium	Myfortic	Inhibitor of IMPDH	Transplantation
Leflunomide	Arava	Inhibitor of DHODH	RA
Teriflunomide (A77 1726)		Active metabolite of leflunomide	MS (phase III)
Gusperimus (15-deoxyspergualin)	Spanidin		Transplantation
Methotrexate	Rheumatrex	Folate antagonist, inhibits dehydropholate reductase	RA, cancer, autoimmune disease
Cell surface molecules, lymphocyte depleting antibodies			
Anti-lymphocyte globulin	Atgam, ALG	Depleting pAb, raised in horses or rabbits against thymocytes	Aplastic anemia, GVHD
Anti-thymocyte globulin	Thymoglobulin, ATG	Depleting anti-T cell pAb, raised in rabbits	Transplantation (induction or treatment of rejection)
Muronomab (OKT3)	Orthoclone (OKT3)	Mouse anti-human CD3 mAb (to the CD3ε chain)	Acute rejection, GVHD
Teplizumab (hOKT3γ1(Ala-Ala), MGA031)		Humanized OKT3 mAb	Type 1 diabetes (phase III)
Visilizumab	Nuvion	Humanized anti-CD3 mAb, non-FcR binding	Ulcerative colitis, Crohn's disease, GVHD (all in clinical trials)
Otelixizumab (ChAglyCD3, TRTX4)		Humanized anti-CD3 mAb (to the CD3ε chain)	Type 1 diabetes, autoimmune disease (phase 3)
Zanolimumab (HuMax-CD4)		Human anti-CD4 mAb	T lymphoid malignancies (phase III)
Siplizumab (MEDI-507)		Humanized anti-CD2 mAb	Psoriasis (phase II): T lymphoid malignancies (phase I)

Reagent[1]	Trade name	Mechanism of action	Status[2]
Alemtuzumab	Campath	Humanized anti-CD52 mAb	B lymphocyte chronic lymphocytic leukemia, (cutaneous) T cell lymphoma: in phase III for MS and transplantation
Ofatumumab (HuMax-CD20)	Arzerra	Human anti-CD20 mAb	B cell chronic lymphocytic leukemia and other B lymphoid malignancies: in clinical trials for autoimmune disease
Rituximab	Rituxan, MabThera	Chimeric anti-CD20 mAb	B lymphoid malignancies, RA: clinical trials in autoimmune diseases, Ab rejection in transplantation
GA101		Humanized anti-CD20 mAb	B lymphoid malignancies (phase III)
Epratuzumab		Humanized anti-CD22 mAb	Autoimmune disease (SLE) (phase III)
CAT-8015		Fusion protein between anti-CD22 mAb and pseudomonas exotoxin A	B lymphoid malignancies (phase II)
Galiximab		Chimeric anti-CD80 mAb (human-monkey)	B lymphoid malignancies (phase III)
Denileukin diftitox	Ontak	Fusion protein between IL-2 and diphtheria toxin	T lymphoid malignancies, malignant melanoma
Cell trafficking and adhesion			
Fingolimod (FTY720)	Gilenia	Sphingosine-1 phosphate receptor modulator	MS
CCX282-B	Traficet-EN	CCR9 inhibitor	Crohn's disease (phase III)
Natalizumab	Tysabri	Humanized anti-α_4 integrin mAb	MS, Crohn's disease
Alefacept	Amevive	Fc fusion protein of extracellular portion of LFA-3: blocks CD2	Psoriasis; GVHD (phase III); kidney transplantation (phase II)
T cell receptor signaling			
Ciclosporin	Gengraf, Neoral, Sandimmune	Inhibitor of calcineurin	Transplantation, RA, autoimmune disease
Voclosporin (ISA247)	Luveniq	Inhibitor of calcineurin	Uveitis (registration), phase III for psoriasis, phase II for transplantation
Tacrolimus (FK506)	Prograf (topical application: Protopic)	Inhibitor of calcineurin	Transplantation, atopic dermatitis, RA, MG, GVHD

Table 1 *(continued)*

Reagent[1]	Trade name	Mechanism of action	Status[2]
Sotastaurin (AEB071)		Inhibitor of PKC	Transplantation (phase II)
Costimulatory blockade			
Abatacept (CTLA4-Ig)	Orencia	Fc fusion protein of extracellular domain of CTLA-4, blocks CD28-CD80/86	RA, in clinical trials for autoimmune disease
Belatacept (LEA29Y)		Fc fusion protein of extracellular domain of CTLA-4, blocks CD28-CD80/86	Transplantation
B-lymphocyte signaling			
Belimumab	Benlysta, LymphoStat-B	Human anti-B lymphocyte stimulator (BLyS) mAb; blocks binding of BLys to its receptor(s)	SLE (registration); RA (phase II); kidney transplantation (phase II)
Atacicept (TACI-Ig)		Fusion protein containing the extracellular ligand-binding portion of the transmembrane activator and CAML interactor (TACI): binds BLys (CD257) and a proliferation inducing ligand (APRIL, CD256)	SLE (phase III); RA (phase II); MS (phase II)
Cytokines/cytokine signaling			
Daclizumab	Zenapax	Humanized anti-CD25 mAb (CD25α chain)	Transplantation: MS (phase 3)
Basiliximab	Simulect	Chimeric anti-CD25 mAb (CD25α chain)	Transplantation
Tasocitinib (CP690,550)		JAK3 inhibitor	RA (phase III), transplantation
Sirolimus	Rapamune	mTOR inhibitor	Transplantation, GVHD
Everolimus	Certican or Zortress (transplantation), Afinitor (oncology)	mTOR inhibitor	Transplantation, renal cell carcinoma: pancreatic neuroendocrine tumors (phase III)
Ridaforolimus		mTOR inhibitor	Metastatic soft-tissue and bone sarcomas (phase III); endometrial cancer, prostate cancer and non-small cell lung cancer (phase II)

Reagent[1]	Trade name	Mechanism of action	Status[2]
Ustekinumab (CNTO1275)	Stelara	Human anti-IL-12/IL-23 mAb	Psoriasis
Tocilizumab (MRA)	Actemra	Humanized anti-IL-6 receptor mAb	RA
Infliximab	Remicade	Chimeric anti-TNF-α mAb	Psoriasis, Crohn's disease, ankylosing spondylitis, psoriatic arthritis, RA, ulcerative colitis
Adalimumab	Humira	Human anti-TNF-α mAb	RA, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, psoriasis, juvenile idiopathic arthritis
Golimumab	Simponi	Human anti-TNF-α mAb	RA, psoriatic arthritis, ankylosing spondylitis
Certolizumab	Cimzia	Humanized anti-TNF-α mAb	Crohn's disease, RA
Etanercept	Enbrel	Fc fusion protein of extracellular domain of TNF receptor 2: binds TNF-α	RA, juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, psoriasis
Pegsunercept		Pegylated TNF receptor 1: binds TNF-α	RA (phase III)
Anakinra	Kineret	IL-1 receptor antagonist	RA

[1]The inclusion of 'tuzu' in the name of the reagent generally indicates an application in oncology, and that of 'lizu' an application as an immune modulator. The inclusion of 'cept' indicates that the product is a fusion protein.
[2]Pharmaceuticals in clinical development are presented with the stage of clinical trials; if not otherwise specified, the agents are marketed for the indication shown.

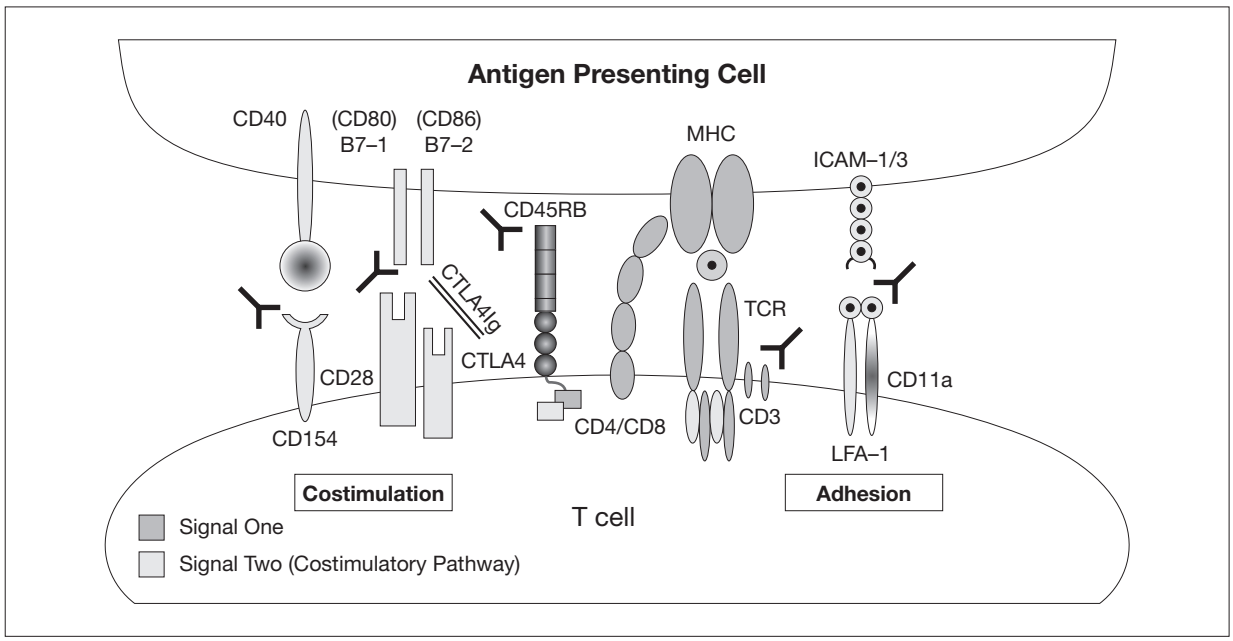

FIGURE 3. TARGET MOLECULES ON THE SURFACE OF T CELLS AND APC INVOLVED IN THE POTENTIAL INDUCTION OF T CELL ACTIVATION AND IMMUNOLOGICAL TOLERANCE

T cell stimulation requires interaction between the TCR and the antigenic epitope in combination with costimulation via a number of cell surface interactions (depicted are those between CD40 and its ligand CD154, and between CD80/CD86 and CD28/CTLA-4). Inhibition of costimulation can result in an anergic state and subsequent unresponsiveness or tolerance. Inhibition of molecular interactions in cell adhesion promotes inhibition of T cell activation (the interaction between LFA-1 and ICAM1/3 interaction is depicted, others include that between VLA-4 and ICAM/VCAM). Also depicted are mAb used to interfere with these interactions. Modified from Vincenti, 2002 (see selected reading).

the case of bronchiolitis obliterans, appear to be useful. Also, some new immunosuppressants, such as mTOR INHIBITORS and mycophenolic acid (MPA) derivatives (see also chapter C11), are claimed to be effective, as these not only contribute to better IMMUNOSUPPRESSION (diminished host attack), but also inhibit the vascular response due to their inherent mechanism of action.

Since ALLOGRAFT rejection is a T cell-mediated process, the main TARGET for IMMUNOSUPPRESSION in transplantation is the T LYMPHOCYTE. But other cell types of the IMMUNE SYSTEM, like B LYMPHOCYTES and MACROPHAGES, are also involved. The role of NK cells in graft rejection is still unclear. This might change in the future when XENOTRANSPLANTATION (transplantation of non-human organs into humans) is likely to become available. The rejection of XENOGRAFTS cannot be prevented by T cell immunosuppressants since it does not solely involve T cells, but also B LYMPHOCYTES, which, in addition to T cell-dependent Ab generation, can be triggered in a T cell-independent way. Ab-mediated rejection has been identified as a major hurdle in preclinical modeling of solid organ XENOGRAFTS, but appears to be less relevant for tissue or cell XENOGRAFTS [9, 10]. Ab-mediated rejection is also relevant in ALLOTRANSPLANTATION because B cell reactivity appears to be involved in CHRONIC REJECTION as well. Some specific anti-B cell drugs have been developed for AUTOIMMUNE DISEASES or B lymphoid malignancies, and a number of immunosuppressants discussed below show both T and B cell inhibitory activity.

Calcineurin inhibitors

Ciclosporin

CsA is a cyclic undecapeptide isolated from the fungus *Tolypocladium inflatum gams*. Its biological activity *in vivo* was first discovered in 1973 in a large microbiology screening program, which included Ab formation to sheep red blood cells (SRBC) in mice. Subsequently, it showed EFFICACY in kidney ALLOTRANSPLANTATION in rats and pigs. This was followed by first clinical trials in human kidney transplant patients, and its introduction to clinical transplantation in 1983 [11]. The International Nonproprietary Name (INN) for the drug that was subsequently developed is CICLOSPORIN. Other names that have been used include cyclosporine (US Adopted Name, USAN) and cyclosporin (former British Approved Name, BAN). Since CsA is a highly lipophilic molecule, it is poorly soluble in water. For oral administration, the first commercial formulation was an oil-based formulation manifesting quite variable absorption. Besides a huge intra-individual variation, there is a large inter-individual variability in response to CsA, i.e., the existence of 'absorbers' and 'non-absorbers' necessitated a drug exposure test in each patient before transplantation. Since the drug shows a relatively narrow therapeutic window, the kidney being the first TARGET for undesirable side effects, drug level monitoring proved necessary to control drug exposure in the therapeutic range. Instead of 16- or 24-hour trough levels, so-called C2 MONITORING (levels at 2 hours after administration) has been introduced as an improved estimate of total exposure. A microemulsion formulation (Neoral) has been marketed since 1995. This formulation shows improved absorption and far less inter- and intra-individual variation.

CsA shows immunosuppressive activity in a large spectrum of animal models of human immune-mediated diseases [12]. Its suppressive effect is mainly restricted to T LYMPHOCYTES. T cell-independent B cell responses are not affected. At the time of introduction to the market, the mechanism of action of CsA was largely unknown. A first insight into its mode of action came from the observation that the compound inhibits the production of IL-2, one of the first CYTOKINES produced after T cell activation. Subsequently, it was demonstrated that CsA inhibits IL-2 gene transcription by interfering with a Ca-dependent intracellular signaling mechanism [13]. A family of cytoplasmic proteins called cyclophilins (CYP) has been identified, and shown to strongly bind to CsA. CYP are enzymes catalyzing *cis-trans* isomerization of peptidyl-prolyl bonds, i.e., so-called proline isomerase or rotamase activity, which is important for proper folding of newly synthesized proteins. This inhibition of rotamase activity, however, does not cause the immunosuppressive effect that is actually mediated by the binding of the CsA-CYP complex to the serine/threonine phosphatase calcineurin, which plays a pivotal role in Ca-dependent intracellular signaling.

Activation of T cells *via* the TCR results in a cascade of events that, among others, involve the activation of the protein tyrosine kinases (PTKs) $p56^{lck}$, $p59^{fyn}$, and ZAP, followed by phosphorylation of phospholipase Cγ1, resulting in generation of second messengers PIP3 and DAGl. These, in turn, result in an increase in cytoplasmic free Ca^{2+} and activation of PKC. Free Ca^{2+} upon complexing with calmodulin, activates the phosphatase, calcineurin. The CsA-CYP complex, upon binding to calcineurin, inhibits its phosphatase activity. Calcineurin dephosphorylates NFAT, which is then translocated to the nucleus where it initiates, together with other transcription factors (e.g., NF-κB and AP-1), expression of early T cell activation genes, especially the gene encoding IL-2 (Fig. 2). CYPs and calcineurin are abundantly expressed in different cell types. The apparent T cell selectivity of CsA has therefore been related to the fact that Ca-dependent T cell activation *via* the TCR uniquely involves the calmodulin-calcineurin pathway. There is as yet no unequivocal proof that potential side effects, like damage to kidney tubules, result from the same intracellular mechanism.

In keeping with its mechanism of action, Ca-independent cell triggering is not affected by CsA. For instance, T cells can be activated *via* the costimulatory CD28 molecule on the cell surface (Figs. 2 and 3), which, in combination with activated PKC, causes lymphokine gene transcription and T cell activation

in the absence of calcineurin activation. It has therefore been hypothesized that this pathway is involved in T cell activation and ALLOGRAFT rejection that is resistant to CsA treatment. Also, inhibition of T cell activation and growth factor-induced cell proliferation by MTOR INHIBITORS targets a pathway that differs from calcineurin inhibition, a phenomenon that underlies the synergy in IMMUNOSUPPRESSION between CALCINEURIN INHIBITORS and MTOR INHIBITORS.

There have been many attempts to identify CsA analogues with an improved therapeutic window, but such attempts have been largely unsuccessful. Presently, one compound structurally similar to CsA, voclosporin (ISA247, Fig. 1), is in the registration phase for uveitis [14]. The compound differs from CsA in the first amino acid residue of the molecule, and has been claimed to be more potent than CsA, while having fewer adverse side effects, in particular renal side effects. The compound was also evaluated in phase II transplantation trials in which a similar EFFICACY and reduced incidence of post-transplant diabetes was noted.

Tacrolimus (FK506)

TACROLIMUS [15,16] is a macrocyclic lactone isolated from the actinomycete *Streptomyces tsukubaensis*. It was discovered in 1984 in an immunological screening program that was specifically established to identify immunosuppressive compounds. Subsequently, its immunosuppressive activity was demonstrated in various animal models of transplantation (rat, dog). The spectrum of immunosuppressive activity appeared to be identical to that of CsA but, remarkably, at much lower doses than CsA, both *in vitro* and *in vivo*. Also, therapeutic drug levels in the circulation appear to be much lower than those for CsA. TACROLIMUS is very lipophilic and poorly soluble in water, resulting in variable absorption, necessitating regular drug monitoring. For oral administration, a solid dispersion formulation in hydroxypropylmethyl cellulose is used. Major side effects are strikingly similar to those of CsA, but also involve the CENTRAL NERVOUS SYSTEM as a TARGET organ. The difference from CsA may at least in part be explained by differences in pharmacokinetics and tissue distribution, as the mechanism of action appears to be the same, i.e., inhibition of calcineurin (Fig. 2). This does not hold for other side effects, like the lack of gingiva hyperplasia and hirsutism (adverse side effects of CsA) and the more pronounced diabetogenic effect of TACROLIMUS.

The similarity between TACROLIMUS and CsA regarding immunosuppressive activity is based on the fact that both drugs inhibit calcineurin phosphatase activity and subsequent intranuclear events in T cell activation. However, in accord with the different molecular structure (Fig. 1), TACROLIMUS does not bind to CYP in the same way as CsA. A family of CYPs with rotamase activity has been identified, the FK506-binding proteins (FKBPs). Of these, FKBP12 appears to be most relevant; upon binding to TACROLIMUS, the complex binds to calcineurin at the same site as the CsA-CYP complex and thereby inhibits T cell activation.

The EFFICACY of TACROLIMUS in humans was first shown in liver transplantation [16]. In liver disease, e.g. liver ALLOGRAFT dysfunction, the absorption of TACROLIMUS is increased and its metabolism decreased, resulting in higher exposure, which actually is required in cases of rejection. Nowadays, the drug is widely used in patients who poorly tolerate CsA; in addition to this conversion in immunosuppressive regimen, the drug is also used in patients directly after transplantation (so-called *DE NOVO* TREATMENT). Interestingly, despite its adverse side effect on pancreas function, TACROLIMUS (together with the MTOR INHIBITOR sirolimus, and the anti-CD25 mAb DACLIZUMAB in INDUCTION TREATMENT) is part of the immunosuppressive regimen used in clinical islet cell transplantation for patients with type 1 diabetes [17]. This steroid-free immunosuppressive regimen was proposed to bypass potential pancreatoxicity of corticosteroids, and was introduced in 2000 as an innovative regimen and boosted islet cell transplantation towards clinical application.

Ascomycin is an immunosuppressant with a similar structure to TACROLIMUS. Ascomycins were first identified as FERMENTATION products of *Streptomyces hygrosopicus* almost 50 years ago. Ascomycin derivatives such as pimecrolimus are currently available for TOPICAL administration in skin diseases [18].

Protein kinase C inhibitor

The PKC family of serine/threonine kinases has a central function downstream of T cell stimulation, *via* the TCR and CD28 costimulatory pathway, and affects the NFAT and NF-κB nuclear TRANSACTIVATION pathways. Three PKC categories exist based on their need for cofactors, described as conventional or classical isoforms, novel isoforms and atypical isoforms. Three isoforms, PKC α, β, and θ, are relevant for intracellular signaling in T and B LYMPHOCYTES. PKC θ activity is mainly restricted to T LYMPHOCYTES. Sotrastaurin (AEB071) selectively inhibits the novel isoforms of PKC α, β, and the classical isoforms PKC δ, ε, η, and θ. Its immunosuppressive activity has been demonstrated in animal models of transplantation [19, 20]. Since PKC inhibition is independent of the calcineurin pathway and the mTOR pathway in T cell activation, the compound shows synergism in IMMUNOSUPPRESSION with CALCINEURIN INHIBITORS (CsA) and MTOR INHIBITORS (everolimus). Synergy with the sphingosine-1 phosphate RECEPTOR modulator fingolimod has also been demonstrated in rodent transplant models. The compound is in advanced clinical development as an immunosuppressant for kidney transplantation, in which it was found to be well tolerated; gastrointestinal disorders and headache were the major adverse side effects. Launch is expected in 2014.

Inhibitors of the mammalian target of rapamycin

Sirolimus and everolimus

Sirolimus is a macrocyclic lactone, isolated from the actinomycete *Streptomyces hygroscopicus*, with a long history dating from the mid 1970s [21]. It was first discovered as an antifungal compound in a soil sample from Easter Island, a Polynesian island in the southeastern Pacific Ocean, also known as "Rapa Nui", which resulted in the name 'rapamycin'. The compound was not further developed because side effects were encountered, including involution of lymphoid tissue. Subsequently, antitumor effects were documented, as well as immunosuppressive activity in rat models of AUTOIMMUNE DISEASE. After structural similarities between sirolimus and TACROLIMUS were identified, studies on the EFFICACY of the compound in rat and mouse organ ALLOGRAFT models were initiated and first reported in 1989. Since then, the compound has been developed for clinical use in transplantation and was launched in 1999. A major complication was the development of a proper oral formulation with acceptable stability, BIOAVAILABILITY and predictability in absorption characteristics. The compound is very lipophilic and poorly soluble in water. In oily solution, as well as in microemulsion, the drug appears to be readily absorbed after oral administration. Another MTOR INHIBITOR with improved physicochemical characteristics, everolimus [22, 23], was identified in a chemical derivatization program, and followed sirolimus with a launch in Europe in 2003 and in the USA in 2010. The relatively narrow therapeutic window requires regular drug exposure monitoring. Major side effects are hyperlipidemia, followed by impaired wound healing, thrombocytopenia, mouth ulcers and cholesterolemia.

MTOR INHIBITORS proved to be extremely potent immunosuppressants, affecting both T LYMPHOCYTES and Ab production by cells in the B lymphocyte lineage. This was demonstrated in a wide spectrum of experimental animal models and in clinical trials in renal transplantation. IMMUNOSUPPRESSION is achieved at relatively low blood levels. In animal models, the drugs show synergistic action in combination with CsA, which points to a difference in mechanism of action. Indeed, whereas CsA and TACROLIMUS inhibit early events in T cell activation, e.g., the expression of GROWTH FACTORS such as IL-2 in G_0-G_1 stage of the cell cycle, MTOR INHIBITORS affect the progression of G_1 to S phase in the cell cycle. At the molecular level, it has been demonstrated that these compounds bind, similar to TACROLIMUS, to CYPs of the FKBP family, in particular FKBP12. However, the complex with FKBP does not bind to calcineurin but to mTOR (alternative names in literature are FRAP, FKBP-rapamycin associated protein; and RAFT, rapamycin and FKBP TARGET), a protein with kinase activity. This resultant complex inhibits intracellular cytokine-driven cell

proliferation, presumably *via* p70 S6 kinase, which is involved in translational control. Apparently, this pathway is particularly relevant in lymphoid cells, underlying the peculiar immunosuppressive characteristics of the drug.

Since growth factor-driven cell proliferation applies to other cell types as well, the therapeutic window of compounds in the class of mTOR INHIBITORS is expected to be narrow. On the other hand, the antiproliferative action of the drug could be beneficial in CHRONIC REJECTION of solid organ allografts. Indeed, it has been shown that rapamycin, in contrast to CsA and TACROLIMUS, inhibits the proliferation of smooth muscle cells *in vitro*, and in animal transplantation models inhibits intima proliferation of blood vessels as observed in CHRONIC REJECTION. Such a beneficial effect has also been documented in the clinical situation of patients with an organ ALLOGRAFT [24]. Recently, this beneficial effect on vasculopathies has gained a new application, i.e., in relation to stents placed in the vasculature of patients after balloon coronary angioplasty. Such stents can show restenosis, resulting in recurrence of the vessel occlusion. Either treatment of the patient with sirolimus, or bathing the stent before implantation in a rapamycin-containing medium, reduces this complication [25, 26]. Interestingly, clinical trials in organ transplantation revealed that the incidence of post-transplant malignancies was lower for patients receiving mTOR INHIBITORS [27], which is essentially in accordance with the original research and discovery of rapamycin as an anticancer drug in the 1980s. The regained interest in mTOR INHIBITORS as potential anticancer drugs [28] resulted in the launch of everolimus for the treatment of advanced renal carcinoma in 2009. Another rapamycin derivative, ridaforolimus, is in advanced stage of development for the indications metastatic soft-tissue and bone sarcomas, as well as advanced endometrial cancer, prostate cancer and non-small cell lung cancer.

Janus kinase 3 inhibitor

JAKs are tyrosine kinases involved in intracellular signaling after stimulation of a broad variety of cell surface RECEPTORS, in particular those of the cytokine RECEPTOR common γ chain family. Cell activation by CYTOKINES *via* their cell surface RECEPTOR results in clustering of RECEPTOR chains and recruitment of the inactive cytosolic form of JAK to the γ chain, which is followed by phosphorylation of tyrosine-based docking sites for STATs. Phosphorylated STATs in turn dimerize and migrate to the nuclei where they stimulate gene transcription. In this respect, JAKs play a major role in the so-called signal 3 pathway of T cell activation, similar to but distinct from mTOR-mediated intracellular signaling (calcineurin/CYP representing signal 1, and COSTIMULATION representing signal 2). This signal 3 pathway involves CYTOKINES like IL-2, IL-4, IL-9, IL-15 and IL-21. There are four JAKs in mammalian cells (JAK1–3 and tyrosine kinase 2), of which JAK3 is restricted to hematopoietic cells and mediates cytokine signaling only *via* RECEPTORS that contain a γ chain. Within the hematopoietic cell lineage, JAK3 shows high expression in NK cells and thymocytes, but not in resting T cells. It is inducible in T cells, B cells and myeloid cells. Therefore, inhibition of JAK3 could be associated with IMMUNOSUPPRESSION without the adverse side effects observed on inhibition of the signal 1 pathway. Tasocitinib (CP690550) is a specific inhibitor of JAK3, discovered in a screen for inhibitors of catalytic activity using a glutathione-S transferase fusion protein with the kinase domain of human JAK3. In rodent and non-human primate transplantation models, the compound showed EFFICACY in IMMUNOSUPPRESSION [29]. This was confirmed in clinical trials in kidney transplantation, in which the drug proved to be well tolerated. In a high-dose group, a higher incidence of infections, cytomegalovirus disease and BK virus nephropathy was noted, and in accordance with the documented presence of JAK3 in NK cells, the latter were strongly reduced [30]. The drug is presently in advanced clinical development for RA.

Cytotoxic drugs

The designation 'CYTOTOXIC DRUGS' is used here to describe drugs that directly interfere with DNA/RNA synthesis and as such affect cell proliferation (see also chapter C11).

Cyclophosphamide

CY is one of the oldest drugs used as an immunosuppressant. It is an ALKYLATING AGENT that was originally used as an anticancer drug. The compound inhibits cells from entering the S phase of the cell cycle, which is subsequently blocked at the G_2 phase. The drug was used in initial trials in clinical transplantation around 1970, in particular in patients with AZA toxicity. Severe side effects were encountered, mainly BONE MARROW depression with severe leukopenia and anemia. Since the introduction of more selective T cell immunosuppressants, the drug has rarely been used because of these side effects. Interestingly, CY was part of a conditioning regimen used in TOLERANCE induction by mixed hematopoietic chimerism (see below). However, CY is among the most powerful inhibitors of B LYMPHOCYTES, and has received renewed interest in experimental animal research in XENOTRANSPLANTATION.

Methotrexate

MTX is a folate antagonist, which nowadays is mainly used in a low-dose treatment regimen (weekly administration) in subsets of patients with RA. Its mechanism of action, under these conditions, has not been completely resolved. It has been suggested that MTX, at a low dose, is converted into a polyglutamate that inhibits transmethylation reactions, resulting in an increased release of adenosine and decreased synthesis of guanine. Although this condition affects purine metabolism, an anti-inflammatory signal is delivered by the binding of adenosine to specific adenosine (A2) RECEPTORS. Thus, MTX, at a low dose, does not appear to be an immunosuppressant, but rather an anti-inflammatory drug.

Azathioprine

AZA has been used since the early days of clinical IMMUNOSUPPRESSION, being introduced as an immunosuppressant for transplantation in 1961. Its development followed the pioneering work on 6-mercaptopurine as an antileukemic agent in the 1940s. Nowadays, the drug is still in use in conjunction with baseline IMMUNOSUPPRESSION (e.g., CsA) in transplantation. Its dosing and dose adaptations are based on adverse side effects, i.e., blood leukocyte counts. Despite the long period of clinical use, its exact mechanism of action is still not completely clear. The drug is converted by red blood cell glutathione to 6-mercaptopurine, which in turn is converted into a series of mercaptopurine containing nucleotides, which interfere with the synthesis of DNA and polyadenylate-containing RNA. One of the nucleotides formed is thioguanylic acid, which can form thioguanosine triphosphate. This can be incorporated into nucleic acids and induce chromosome breaks and also affects the synthesis of coenzymes. As a general inhibitor of cell proliferation, AZA affects both T and B lymphocyte reactivity. Presently, there is an intention to minimize the use of AZA, or restrict its use to treatment of rejection episodes, with a proposed replacement by MPA derivatives [31].

Mizoribine

Mizoribine is an imidazole nucleoside originally isolated as a potential antibiotic from the culture filtrate of the soil fungus *Eupenicillium brefeldianum*. Its immunosuppressive activity was demonstrated first by the inhibition of mouse lymphoma cell lines and subsequently by the inhibition of an Ab response in mice immunized with SRBC. It was subsequently shown that the drug is phosphorylated intracellularly to the active form, mizoribine 5'monophosphate, under the influence of adenosine kinase. This compound is a competitive inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH), which is a rate-limiting enzyme in purine biosynthesis in lymphoid cells (Fig. 4). The drug has been in use in clinical transplantation since 1984, only in Japan, mainly as a replacement for AZA [32]. It is also proposed for the treatment of various renal diseases, including IgA nephropathy, lupus nephritis, and nephritic syndrome [33].

Mycophenolic acid

Mycophenolate mofetil (RS-61443) is the morpholinoester of MPA, a FERMENTATION product of various

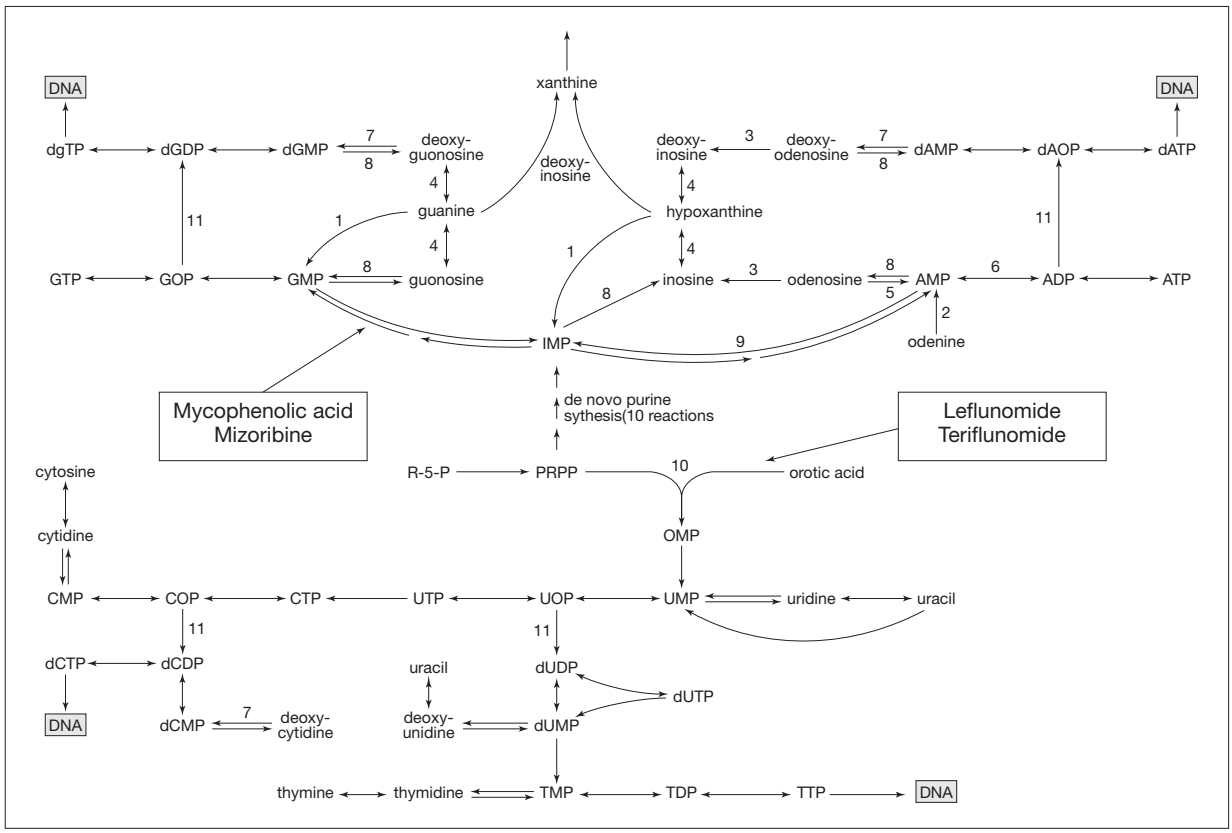

FIGURE 4. THE PURINE/PYRIMIDINE PATHWAY AND SITES OF INHIBITION BY MPA, MIZORIBINE, LEFLUNOMIDE, AND TERIFLUNOMIDE

For purine metabolism, two pathways are shown, the salvage pathway [conversion of guanine to GMP, mediated by hypoxanthine-guanine phosphoribosyltransferase (HGPRT)] and the de novo *pathway [conversion of IMP to GMP, mediated by inosine monophosphate dehydrogenase (IMPDH)]. For pyrimidine metabolism, the* de novo *pathway (conversion of orotic acid to UMP) and the salvage pathway (conversion of uridine to UMP, and cytidine to CMP) are shown. Major enzymes involved are HGPRT (1), adenine phosphoribosyltransferase (2), adenosine deaminase (3), purine nucleoside phosphorylase (4), adenosine kinase (5), adenylate kinase (6), deoxycytidine kinase (7), 5′nucleotidase (8), adenosine monophosphate dehydrogenase (9), dihydroorotate dehydrogenase (DHODH) (10), and ribonucleotide reductase (11).*

Penicillium species, originally isolated and purified in the early 1910s. MPA was originally studied for its antibacterial and antifungal activity, and subsequently for its antitumor activity in the late 1960s, but these activities were not further followed in clinical development. The compound was specifically selected as a drug that inhibited IMPDH in the mid 1980s. This selection was based on the fact that MPA was not a nucleoside, that it did not require phosphorylation to become active, and that it did not show the unwanted side effects of nucleosides, such as induction of chromosome breaks and inhibition of DNA repair enzymes. MPA is a non-competitive reversible inhibitor of IMPDH (Fig. 4). Mycophenolate mofetil was developed as an immunosuppressant. It is rapidly hydrolyzed by esterases to yield MPA. The drug was

introduced to the market in 1995 for the transplantation indication, mainly as a replacement for AZA. Major side effects are gastrointestinal intolerability and BONE MARROW depression, documented for a marketed formulation (Cellcept). An enteric-coated formulation of mycophenolate sodium (Myfortic), with fewer gastrointestinal side effects, was approved for transplantation in 2004.

The fact that inhibition of IMPDH causes quite selective IMMUNOSUPPRESSION is due to the relevance of different pathways of purine metabolism in different cell types (Fig. 4). Two pathways exist, the salvage pathway (conversion of guanine into GMP by hypoxanthine-guanine phosphoribosyltransferase, HGPRT) and the *de novo* pathway (conversion of IMP into GMP mediated by IMPDH). LYMPHOCYTES depend highly on the *de novo* pathway and do not use the salvage pathway. At the other end of the spectrum, cells of the CENTRAL NERVOUS SYSTEM depend highly on the salvage pathway. Cell types like smooth muscle cells, fibroblasts, endothelial cells and epithelial cells can use both pathways for purine synthesis. Hence, inhibition of IMPDH results in a quite selective inhibition of purine biosynthesis in LYMPHOCYTES. For MPA, an additional selectivity has been documented for the two isoforms of IMPDH; the type I isoform is predominantly expressed in resting LYMPHOCYTES and the type II is strongly expressed in LYMPHOCYTES after activation. This type II isoform is four to five times more sensitive to inhibition by MPA than the type I isoform. Hence, MPA is a more potent inhibitor of activated LYMPHOCYTES. As both T and B LYMPHOCYTES are affected by IMPDH inhibition, MPA is an effective inhibitor of both T and B LYMPHOCYTES, like mizoribine mentioned above. This has not only been demonstrated in rodent models, but also in pig-to-primate XENOTRANSPLANTATION models, where the suppression of xeno-Ab formation is a critical issue.

Apart from affecting RNA synthesis, IMPDH inhibition has other effects. IMPDH inhibition results in depletion of GTP in LYMPHOCYTES (Fig. 4), which affects the transfer of fucose and mannose to glycoproteins. This can affect ADHESION MOLECULES on the cell surface. Examples of ADHESION MOLECULES whose expression is inhibited are VLA-4 and ligands for SELECTINS. On the basis of this mechanism, IMPDH inhibitors could affect the recruitment of inflammatory cells into tissue, and effector-TARGET cell interactions within tissues. This potential effect has been demonstrated by the inhibition of the adhesion of T LYMPHOCYTES to endothelial cells *in vitro* when either T cells or endothelial cells are pretreated with MPA.

Finally, as IMPDH inhibition can affect cell growth in other cell types besides LYMPHOCYTES, it might have an effect in CHRONIC REJECTION. MPA inhibits proliferation of human smooth muscle cells *in vitro*, and is effective in a rat vessel transplantation model that mimics CHRONIC REJECTION in solid organ allografts. At present, MPA derivatives are mainly used in the transplantation setting; besides being a potential replacement for AZA, the drug has also been studied as a replacement for CALCINEURIN INHIBITORS [34]. Potential applications in AUTOIMMUNE DISEASES, such as SYSTEMIC LUPUS ERYTHEMATOSUS (SLE), myasthenia gravis and glomerular disorders, are in an exploratory phase [35].

Leflunomide

LEFLUNOMIDE is an isoxazole derivative, originally synthesized as part of an agriculture herbicide program in the mid 1970s. Its anti-inflammatory activity was demonstrated in animal models of adjuvant arthritis and experimental allergic encephalomyelitis. First studies on its immunosuppressive action in models of AUTOIMMUNE DISEASE were documented in 1990. Since then, the compound has been extensively investigated in animal models of solid organ allo- and XENOTRANSPLANTATION (rodents, dog, non-human primates) and proved to be a potent immunosuppressant both for T and B LYMPHOCYTES. LEFLUNOMIDE was subsequently developed as a so-called disease-modifying antirheumatic drug (DMARD) for RA and was launched in 1998 [36,37]. Following this launch, a number of clinical trials have shown the EFFICACY of the compound in transplantation. A major drawback for its development for the transplantation indication is the long $t_{½}$ in man (15–18 days). Therefore, analogues with a shorter $t_{½}$ (malononitrilamides) have been explored as potential immunosuppres-

sants, but these analogues failed in clinical trials. The active metabolite of LEFLUNOMIDE, teriflunomide (A77 1726) is currently in advanced clinical development for MULTIPLE SCLEROSIS (MS) [38].

LEFLUNOMIDE is a produg, which under the influence of the intestinal mucosa or liver, is metabolized to the active isoxazole open-ring form (compound A77 1726). The antiproliferative action (entry into S phase of the cell cycle), although still not completely understood, is based on its inhibition of two different intracellular pathways. The first involves the enzyme dihydroorotate dehydrogenase (DHODH), which is the fourth rate-limiting sequential enzyme in the *de novo* pyrimidine biosynthetic pathway (Fig. 4). This pathway is particularly relevant for the proliferative response of LYMPHOCYTES, as limited intracellular pools of substrates restrict the use of the salvage pathway by these cells. Also, the intracellular concentration of DHODH is relatively low in lymphoid cells, so that this pathway is easily inhibited. Inhibition of DHODH results in depletion of intracellular pyrimidine nucleotides, which have several vital cellular functions, including synthesis of DNA, RNA, glycoproteins (adhesion proteins) and phospholipids. Another mechanism of action is inhibition of protein phosphorylation by inhibition of tyrosine kinase activity. The relevance of this mechanism for the immunosuppressive action of LEFLUNOMIDE is questionable as kinase inhibition generally requires higher concentrations *in vitro* than does the inhibition of DHODH. Also, there are claims that LEFLUNOMIDE inhibits the activation of NF-κB.

Like inhibition of IMPDH, inhibition of DHODH affects not only T cells but also B cells. Hence, LEFLUNOMIDE has been shown to be a potent drug affecting T-dependent and T-independent Ab synthesis. There are reports that LEFLUNOMIDE affects B cells even more potently than T LYMPHOCYTES. There are also claims that the drug might be effective in CHRONIC REJECTION, as demonstrated by the *in vitro* inhibition of rat smooth muscle cell proliferation, and its EFFICACY *in vivo* in rodent vessel transplantation, a model mimicking vascular pathology in CHRONIC REJECTION of solid organ allografts. Apart from its immunosuppressive activity, the anti-inflammatory action of the drug (inhibition of the production of INFLAMMATORY CYTOKINES) might be relevant for this indication.

Gusperimus (15-deoxyspergualin)

Gusperimus is a SYNTHETIC derivative of spergualin, an antibiotic isolated from the soil bacterium *Bacillus laterosporus* in a screening program for anticancer drugs in the early 1980s. Its immunosuppressive activity in a mouse skin transplantation model was demonstrated in 1985, and the drug was subsequently developed for use in transplantation. It has been commercially available in Japan since 1994. A major drawback in its clinical application is the low oral BIOAVAILABILITY of the drug, which means that it has to be administered parenterally, and also its instability in aqueous solution. In Europe, gusperimus received orphan drug status in 2001 for the treatment of Wegener's granulomatosis, a serious form of vasculitis frequently associated with permanent disability and/or fatal outcome; a clinical trial showing benefits in these patients has recently been published [39].

Gusperimus is a potent immunosuppressive compound in animal models of transplantation and AUTOIMMUNITY. B cells are equally well inhibited as T cells, and the drug also shows anti-inflammatory activity. However, the mechanism of action is not very well understood. The compound affects the differentiation of stimulated LYMPHOCYTES into EFFECTOR CELLS, e.g., CYTOTOXIC T LYMPHOCYTES (CTLs) or Ab-producing plasma cells, and the entry of cells from G_0 or G_1 into the S phase of the cell cycle. At the molecular level, the drug blocks the nuclear transcription factor NF-κB, which appears to underlie its inhibitory activity in production of proinflammatory CYTOKINES and maturation of DENDRITIC CELLS.

Also, binding of gusperimus to a cytosolic member of the heat shock protein (HSP) family, HSP70, has been described. HSPs participate in the folding and unfolding of proteins and play a role in protein transport to intracellular organelles (the so-called chaperone function of these molecules, for instance in antigen presentation). This capacity of the drug has received attention for a different area, namely parasite infection. HSP mediate a number of processes that are crucial to parasite survival, such as thermoprotection, through their chaperone function, export of parasite proteins to the erythrocyte, parasite interorganelle protein trafficking, and regulation

of parasite infectivity. Gusperimus, by binding HSP70, has therefore been proposed as a new innovative antimalarial drug [40].

Cell trafficking and adhesion

Fingolimod (FTY720)

Fingolimod is a novel immunomodulatory agent that affects lymphocyte recirculation. It is a chemical derivative of myriocin, a metabolite of the ascomycete *Isaria sinclairii*. The compound is an analogue of sphingosine, a major constituent of sphingolipids in the cell membrane that function as lipid signaling molecules. In preclinical studies, the compound prolonged ALLOGRAFT survival with high potency and EFFICACY in mice, rats, dogs and non-human primates, and proved to be effective in other preclinical models of immune-mediated diseases as well [41]. In these models, a high level of synergy with other immunosuppressants like CsA was documented. Also, in contrast to other low molecular weight xenobiotic immunosuppressants, the compound has a rather large therapeutic window. Remarkably, it does not affect protective immunity, for example, to virus infections. Fingolimod was tested in phase III clinical trials in kidney transplantation, but failed to show an additional benefit in the prevention of rejection. The drug was found to be safe; the main adverse side effect was transient and asymptomatic bradycardia, which proved to be a pharmacodynamic effect directly associated with circulating drug concentrations [42]. The drug was subsequently evaluated for the treatment of MS [38, 43], and is approved in 2010.

The mechanism of action is associated with the physiological response of LYMPHOCYTES in immune responses, including the recirculation of T LYMPHOCYTES between blood and lymphoid tissue, which involves contact with antigen, and their entrance into lymph nodes from the blood *via* high endothelial venules or from other locations *via* afferent lymphatics. Signaling of the lymphocytic sphingosine-1-phosphate RECEPTOR by the endogenous LIGAND, sphingosine 1-phosphate, is required for LYMPHOCYTES to exit from lymph nodes. Upon phosphorylation of the drug by sphingosine kinase-2, the resulting phosphate complex binds to four of the five G protein-coupled sphingosine 1-phosphate RECEPTOR subtypes. This causes internalization and degradation of the cell membrane RECEPTOR, which affects the capacity of the cells for recirculation, i.e., exit from lymph nodes. As a result, circulating LYMPHOCYTES, both T and B cells, accumulate in lymph nodes and no longer recirculate to the periphery so that they cannot participate in immune responses [41]. The same mechanism applies to the beneficial action of fingolimod in MS, namely, inhibition of migration of T LYMPHOCYTES from lymph nodes to the CENTRAL NERVOUS SYSTEM and thereby prevention of T cell-mediated local injury. In addition, it has been proposed that the compound inhibits stimulation of neural cells by excess sphingosine 1-phosphate. This stimulation is associated with astrogliosis and perturbed gap junctional communication between cells in the CENTRAL NERVOUS SYSTEM.

Anti-lymphocyte/anti-thymocyte globulin/anti-CD3 Ab

Horse ALG and rabbit ATG [44] were originally introduced as immunosuppressants for INDUCTION TREATMENT or for treatment of rejection episodes. These reagents induce a severe but temporary depletion or inactivation of T cells (in the case of ATG) or LYMPHOCYTES (ALG) from the circulation. ATG appears more effective and is more widely used than ALG, and is more stringent than other INDUCTION AGENTS such as anti-CD25 mAb mentioned below [45]. Side effects include fever, leukopenia and thrombocytopenia, and depending on the patient population, a higher incidence of cytomegalovirus disease is commonly observed.

Muronomab is a mouse IgG2a mAb specific for the CD3 ε-chain of the TCR complex. Upon muronomab binding to CD3, the entire RECEPTOR complex is modulated from the cell surface. This modulation results in depression of T cell activity. Muronomab, in addition, is known to induce APOPTOSIS of the cells, and hence also has T cell-depleting activity. Muronomab was the first mAb to be approved for clinical application in the mid 1980s. Its use in either INDUCTION

TREATMENT or treatment of steroid-resistant rejection has revealed the potential side effects of this class of BIOLOGICALS in general. A major side effect is the so-called cytokine release syndrome that is related to the potent stimulatory activity of the mAb (besides its depressing activity). This cytokine release syndrome can emerge quickly upon first dosing and results in malaise, fever, myalgia, rigors, headache and diarrhea, in more severe cases hypotension, wheezing and/or pulmonary edema. Also a temporary rise in serum creatinine is part of this cytokine release syndrome. A second side effect is related to the fact that muronomab is a mouse Ig, and thus can induce anti-mouse Ab formation. The presence of such Ab in the circulation reduces the EFFICACY of muronomab in subsequent courses of treatment. A number of engineered anti-CD3 Ab have been developed to circumvent this adverse side effect (Tab. 1).

Besides the transplant indication, anti-CD3 Ab are currently being profiled for application in AUTOIMMUNE DISEASES [46], and there are indications that anti-CD3 Ab might induce immune TOLERANCE as well [47]. A promising application has been documented in patients with recent-onset type 1 diabetes, at a time when the autoimmune destruction of pancreatic islets of Langerhans is not complete. In such patients, the mAb teplizumab reduced the loss of islet cell function [48].

Other lymphocyte-depleting Ab

The HUMANIZED anti-CD52 mAb alemtuzumab was originally developed for treatment of graft *versus* host disease (GVHD), and approved for treatment of chronic lymphocytic leukemia and T cell lymphoma. The Ab recognizes a peptide linked to a membrane glycoprotein present on all LYMPHOCYTES, MONOCYTES and MACROPHAGES. It causes a marked and persistent depletion of LYMPHOCYTES. Within the T LYMPHOCYTE lineage, this particularly affects the $CD4^+$ population [49]. Alemtuzumab was subsequently evaluated as an INDUCTION AGENT in kidney transplantation, at much lower dose levels than those used in oncology. Remarkably, it appeared that patients, after this induction, needed much lower doses of CsA during MAINTENANCE TREATMENT, based on which it was suggested that alemtuzumab INDUCTION TREATMENT induced a status of 'almost' TOLERANCE ("prope" TOLERANCE) [50]. This phenomenon, in conjunction with CsA in maintenance IMMUNOSUPPRESSION, was not apparent in subsequent trials in which CsA was replaced by mTOR INHIBITORS. Although the mAb caused long-lasting severe lymphopenia, lymphopenia-associated adverse side effects, such as an increased incidence of infection or post-transplant lymphoproliferative disease, were not apparent [51]. However, the long-lasting lymphopenia is one main reason why the mAb has not gained a strong position in induction IMMUNOSUPPRESSION after transplantation.

Various mAb to T cells subsets have been developed. The HUMANIZED anti-CD2 mAb siplizumab was originally tested as a treatment for GVHD [52], and is presently in clinical trials for psoriasis and in early clinical testing for application in solid organ transplantation and T lymphoid malignancies. Anti-CD4 mAb have been tested in transplant models, but this has not been followed by clinical development: the fully HUMAN anti-CD4 mAB is in phase III trials for treatment of cutaneous T LYMPHOCYTE lymphoma.

A number of mAb to B LYMPHOCYTES, in particular anti-CD20 mAb, have been developed. The HUMANIZED mAb rituximab was the first to enter the market in 1997 as treatment for B cell non-Hodgkin's lymphoma. The mAb is in clinical trials for many other indications, including RA and AUTOIMMUNE DISEASE in which AUTOANTIBODIES play a role (see chapter C15). In the transplantation setting, rituximab is used in the treatment of Ab-mediated rejection and management of ABO blood group incompatibility, but there are no published trials [53]. Other HUMANIZED or HUMAN anti-CD20 MAB have been profiled for an indication in oncology or AUTOIMMUNE DISEASE. Of more recent date are the anti-CD22 Ab, which are similarly in clinical development for AUTOIMMUNE DISEASE or B lymphoid malignancies (Tab. 1).

Anti-IL-2 receptor Ab

The development of mAb to the α-chain of the IL-2R (anti-CD25 Ab) is based on the fact that this

chain of the RECEPTOR is expressed on the surface of T cells only after activation. In peripheral blood, $CD25^+$ cells are present in quite low numbers. Thus, CD25 Ab are presumed to bind only activated T cells. Two mAb have been approved for the transplantation indication during the last decade; one is a HUMANIZED mAb (DACLIZUMAB [54]), and the other a CHIMERIC molecule (BASILIXIMAB [55]). Both mAb reduce the incidence of ACUTE REJECTION after kidney transplantation, and permit dose levels of immunosuppressants to be reduced in the post-transplant period, while maintaining adequate IMMUNOSUPPRESSION. In most patients, INDUCTION TREATMENT with anti-CD25 mAb is sufficient, but in high-risk patients a more stringent INDUCTION REGIMEN including ATG shows a better outcome after transplantation. Interestingly, this appears not to apply for type 1 diabetes patients receiving an islet transplant. In this condition, a more severe INDUCTION TREATMENT using ATG provided a better long-term outcome than INDUCTION TREATMENT using BASILIXIMAB. There are no relevant side effects to anti-CD25 mAb, and $CD25^+$ T cells are absent in the circulation as long as RECEPTOR-saturating Ab levels are maintained. Amongst other indications of the Ab, some recent exploratory trials showed a beneficial effect of DACLIZUMAB on clinical manifestations of MS patients [56].

Ab targeting cytokines or cytokine signaling

While MTOR INHIBITORS and anti-CD25 mAb have been marketed for use in transplantation, a number of BIOLOGICALS have been developed to address CYTOKINES or cytokine signaling in inflammatory conditions. The initial application of these Ab was in autoimmune indications, mostly psoriasis and RA. This seems logical, as in these conditions an inflammatory condition is a main contributor to the disease manifestations, while this is less the case in a transplant recipient. These BIOLOGICALS are included in Table 1. Their use in the transplant setting may be anticipated to reduce inflammatory reactions, such as those observed in the first post-transplant period, in particular in the case of islet cell transplantation. For instance, the use of ETANERCEPT, ANAKINRA and more recently ustekinumab has been proposed for the induction phase after islet transplantation to reduce INFLAMMATION, and also for the TARGET CYTOKINES (IL-1, IL-12) to TARGET their role in antigen presentation.

Ab to adhesion molecules

Therapeutic mAb to ADHESION MOLECULES have been generated either for use in transplantation or for immune diseases like psoriasis. Anti-CD11a (anti-LFA-1) Ab received attention because of their EFFICACY in experimental transplantation models, including non-human primates. Two anti-CD11a mAb were launched, either for the transplantation indication (odulinomab) or for psoriasis (efalizumab). However, these mAb were withdrawn after launch because of severe adverse side effects in a number of patients, specifically progressive multifocal leukoencephalopathy associated with JC virus infection.

The HUMANIZED anti-VLA-4 mAb natalizumab is on the market for MS and Crohn's disease. This mAb has not been evaluated in transplantation, although molecules like VLA-4 or its ligands (VCAM, ICAM) are interesting targets, based on experimental animal data that the blockade of such interactions results in ALLOGRAFT survival.

Alefacept is a dimeric fusion protein consisting of the CD2-binding portion of human LFA-3 linked to the Fc portion of human IgG1, which blocks the interaction between CD2 and LFA-3. This interaction supports COSTIMULATORY SIGNALS in antigen presentation. The fusion protein was approved in 2003 for treatment of psoriasis, and is in advanced clinical development for GVHD (based on the results with the anti-CD2 mAb siplizumab mentioned above), T lymphoid lymphomas and kidney transplantation. A potential application in the transplant setting is evident from data in a non-human primate transplant model, in which alefacept was efficacious in combination with costimulatory blockade [57].

Costimulatory blockade

Since the recognition of COSTIMULATION (signal 2) in antigen presentation (Fig. 3), interest has been intense in the therapeutic application of mAb that block COSTIMULATORY SIGNALS. Anti-CD80 (B7.1) and anti-CD86 (B7.2) mAb showed EFFICACY in a non-human primate transplantation model [58], but have not been developed further. A number of anti-CD154 mAb have been successfully applied in non-human primate transplantation models, but failed in subsequent clinical trials. This was due to the emergence of thromboembolic side effects, which were less evident in animal testing because of the different cellular distribution of the corresponding antigen, being present on human platelets but not on platelets of other species. An anti-CD40 mAb showed EFFICACY in non-human primates [59], and a derivative of this mAb is currently in early development for PSORIATIC ARTHRITIS.

Blocking the CD28-B7 (CD80, CD86) integration with fusion proteins has proven to be very successful, owing to the fact that this pathway is most important in COSTIMULATION. The first product, abatacept (CTLA4-Ig), is a fusion protein between the extracellular domain of the CD28 antagonist. CD152 (CTL antigen, CTLA4, a member of the Ig superfamily expressed on the surface of T helper cells) and an Ig Fc fragment. In costimulatory signaling, CTLA4 transmits an inhibitory signal to T cells, whereas CD28 transmits a stimulatory signal. CTLA4-Ig has 200-fold higher AFFINITY for CD80 than for CD86 and is 100 times more potent in blocking CD80-dependent COSTIMULATION than CD86-dependent COSTIMULATION. The fusion protein showed EFFICACY in a non-human primate transplantation model, either given alone or in combination with anti-CD154 mAb, but TOLERANCE, observed in rodent transplant models using costimulatory blockade, was not observed [60]. Abatacept has subsequently been developed and since 2008 has been marketed for treatment of RA [61]. The choice of RA and not transplantation as a TARGET indication was based, amongst other reasons, on the anticipation that a product with a higher binding avidity might be needed in the more stringent setting of an immune response to a transplant.

In search of a second generation product with a higher binding AFFINITY, a mutagenesis and screening strategy was used to identify high-avidity mutants with slower dissociation rates. Two amino acid substitutions were identified as potentially useful, and this resulted in the development of belatacept (LEA29Y) for transplantation [62]. The drug is now approved for kidney transplantation. Ongoing trials are addressing, amongst others, the effectiveness of the combination between belatacept and an MTOR INHIBITOR in inducing TOLERANCE. Remarkably, very few adverse side effects have been noted in clinical trials, which gives belatacept a substantial advantage over conventional xenobiotic immunosuppressants. In the phase II trial, some patients treated at a higher dose of belatacept developed post-transplant lymphoproliferative disease, which caused some concern. This IMMUNOSUPPRESSION-associated complication did not emerge in other trials.

Limitations of presently available immunosuppressants

Surgical techniques, including the creation of anastomoses between blood vessels, made it possible to introduce solid organ transplantation into the clinic to replace dysfunctional organs in end-stage organ disease. The first kidney transplant was conducted in 1954 [3]. Since the donor and recipient in this pioneering trial were identical twins, organ rejection was not an issue, but the management of graft rejection has been a major focus in making transplantation an acceptable clinical procedure. Initial exploratory investigations were performed using antiproliferative drugs, which are well known for their severe adverse side effects, and corticosteroids. The introduction of the CALCINEURIN INHIBITOR CsA has revolutionized transplantation. The main problem of current baseline immunosuppressants is still toxicity (direct drug toxicity, increased susceptibility to infection, development of tumors). In this regard, the introduction of the antiviral drug ganciclovir (Cytovene) in 1989 for the prevention or treatment of cytomegalovirus infection or disease, is often considered as a major hallmark in clinical transplantation [63].

One way to cope with the systemic side effects of immunosuppressants is to TARGET drugs specifically to the organ involved. An example of this approach comes from lung transplantation, which generally requires strong IMMUNOSUPPRESSION and hence has a rather small therapeutic window for immunosuppressives. Local delivery by inhalation has been evaluated for a number of drugs, including anti-inflammatory agents and immunosuppressants [64]. Administration of glucocorticosteroids by inhalation did not improve the outcome of lung transplantation [65], but for CsA, delivered by aerosol, beneficial results have been reported, not only in ACUTE REJECTION, but also in CHRONIC REJECTION (bronchiolitis obliterans) [66].

A broad armamentarium of immunosuppressants is nowadays available to increase the therapeutic window of immunosuppressive cocktails in INDUCTION TREATMENT and MAINTENANCE TREATMENT after transplantation, and achievements can be illustrated by a number of examples. With regard to INDUCTION TREATMENT, the present experience in renal transplantation indicates that the rather mild INDUCTION TREATMENT with anti-CD25 mAb is sufficient to achieve long-term graft function, but in high-risk patients, a more stringent INDUCTION REGIMEN using ATG is apparently needed [55]. In heart transplantation [1] and also in islet cell transplantation, the outcome in general appears to be better for patients after ATG INDUCTION TREATMENT, despite the fact that ATG INDUCTION TREATMENT is associated with an increased risk for cytomegalovirus disease. Also, a more stringent INDUCTION REGIMEN appears to be associated with a lower dose requirement for immunosuppressants during maintenance, as has been most clearly shown for INDUCTION TREATMENT with the mAb alemtuzumab. However, this mAb can give long-lasting lymphopenia with the associated concerns for infectious disease [50,51]. Improvements in maintenance IMMUNOSUPPRESSION focus on reducing the need of corticosteroids and also to reduce the use of CALCINEURIN INHIBITORS. Targeting COSTIMULATION offers the promise to fulfill this need. Clinical trials with belatacept showed similar EFFICACY outcomes as those with CALCINEURIN INHIBITORS, with a much lowered incidence of adverse side effects [62]. The introduction of a steroid-free immunosuppressive regimen in 2000 (the Edmonton protocol) boosted the development of islet cell transplantation to become a clinical procedure [17]. Interestingly, this protocol included TACROLIMUS, which is well known for its diabetogenic adverse side effects.

The risk of graft dysfunction after transplantation is related to the quality of the donor organ and the potential of the recipient to reject the graft. With regard to the intrinsic potential for graft rejection, patients who have been sensitized to MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) ANTIGENS and have circulating anti-HLA Ab represent a high-risk population. Therefore, patients who are on the waiting list for a transplant are tested for such Ab using a CYTOTOXICITY assay with a panel of different TARGET cells. Often, patients with a high PRA (panel-reactive Ab) score remain longer on the waiting list than patients with a low score. Kidney transplantation in high-risk patients can be managed, on the one hand, by avoiding nephrotoxic drugs, such as CsA and TACROLIMUS, and on the other hand by increasing the strength of induction IMMUNOSUPPRESSION [67]. Most xenobiotic immunosuppressants TARGET intracellular pathways that are more or less shared between different cell types; this phenomenon underlies most of their adverse side effects. It was assumed that biological immunosuppressants, because of their higher level of SPECIFICITY and their targeting of cell surface structures, would have a better therapeutic window. This is not always the case, as exemplified by the severe adverse side effects noted with a number of BIOLOGICALS with immunosuppressive function, e.g., anti-CD154 mAb causing thromboembolic manifestations that resulted in the discontinuation of the clinical development, the HUMANIZED anti-CD20 mAb ocrelizumab showing infections and death in some patients during phase III trials in RA leading to discontinuation of development, and anti-CD11a mAb causing progressive multifocal leukoencephalopathy, resulting in withdrawal from the market. Ocrelizumab is now in phase II for relapsing-remitting MS.

These considerations point to the requirement for a more individually customized immunosuppressive regimen. Drug level assessment and measurement of pharmacodynamic parameters are performed, but only partially meet the requirements. These analyses address drug exposure and not the intrinsic

susceptibility of the patient's IMMUNE SYSTEM to IMMUNOSUPPRESSION nor the extent to which the individual needs to be immunosuppressed to avoid rejection. Presently, initiatives are in progress to change this empirical approach of patient management to a more logical strategy. For instance, this includes the introduction of specific immune monitoring assays before and after transplantation, and the introduction of pharmacogenomics [68–71].

With the improved outcome of solid organ transplantation, providing better management of ACUTE REJECTION episodes, CHRONIC REJECTION is nowadays a major cause of graft failure in the long term after transplantation. Therefore, there is a clinical need for drugs that interfere with this process. Since CHRONIC REJECTION can include Ab-mediated rejection, a beneficial effect of immunosuppressants that TARGET B cells and Ab formation following indirect antigen presentation has been proposed. Indirect antigen presentation includes costimulatory signaling, and it is anticipated that effective blockers of COSTIMULATION will have a beneficial effect on CHRONIC REJECTION. Also, prevention or treatment of CHRONIC REJECTION or graft vessel disease, in addition to improved novel immunosuppressants, may require non-immunological approaches, which are not considered here.

A special category of high-risk patients are those who receive a transplant across the ABO red blood cell barrier. Isohemagglutinins are feared for their potential to cause immediate Ab-mediated rejection of the graft. Therefore, there is a need for Ab management in both the patient with a high PRA score and the patient receiving an ABO incompatible graft. Current strategies include the removal of Ab, e.g., by plasmapheresis and specific extracorporeal immunoadsorption in an acute phase, treatment with intravenous immunoglobulin (IVIG) and treatment with B lymphocyte immunosuppressants [53, 72–74]. IVIG is used in multiple conditions, not only to give protection against infection in patients with hypogammaglobulinemia, but also as treatment of patients with inflammatory disorders or AUTOIMMUNE DISEASE (see chapter C2). The mechanism of action of IVIG in modulation of immune reactivity and INFLAMMATION is not completely clear. At the molecular level, neutralization of anti-HLA Ab, inhibition of CYTOKINES IL-1β, IFN-γ, IL-2 and IL-6, and inhibition of terminal COMPLEMENT COMPONENTS C5b-9 have been proposed, and at the cellular level, regulation of the B lymphocyte REPERTOIRE and activities through binding to FcγRI. The latter includes induction of B lymphocyte APOPTOSIS, maturation of DENDRITIC CELLS and induction of Treg LYMPHOCYTES. There are also claims that IVIG affects NK cell function and the activity of polymorphonuclear GRANULOCYTES in INFLAMMATION [75, 76].

A major issue in suppressing synthesis of Ab is the fact that Ab-producing plasma cells have low expression of cell surface RECEPTORS and are not susceptible to conventional immunosuppressants. In addition, there are no immunosuppressants that specifically TARGET T cell-independent Ab synthesis, i.e., the process following which isohemagglutinins are produced. The anti-CD20 mAb rituximab, which is marketed for B lymphoid malignancies, has been evaluated in the transplant setting. Other anti-B cell mAb, including anti-CD22 mAb and BIOLOGICALS affecting intracellular B cell signaling (belimumab [77] and atacicept [78]), are in registration phase or advanced clinical development, respectively, for AUTOIMMUNE DISEASES (Tab. 1).

New targets

From the preceding section, it is evident that there is a continued need to develop novel innovative immunosuppressants with fewer adverse side effects. This is most likely to be achieved by selecting targets for pharmacological intervention that are specifically relevant for the immune response and not for biological processes in other organ systems. Besides selectivity, a critical aspect in the selection of new drug targets is the redundancy of potential targets. Non-redundancy of targets is demonstrated most convincingly by the phenotype of human primary immunodeficiency and knockout mice. Different activation mechanisms are specific for the IMMUNE SYSTEM and might be relevant for direct and indirect presentation of ALLOGRAFT ANTIGENS.

Antigen presentation to T cells is still an attractive TARGET (Fig. 3), considering the promising results with abatacept and belatacept, and despite the

failure of CD154 mAb. Not only are processes in COSTIMULATION unique to T cell activation, but also it is well established that inhibition of signal 2 in T cell activation has the potential to induce T cell anergy or unresponsiveness, which could lead to antigen-specific TOLERANCE. In particular, the combination between costimulatory blockade and mTOR inhibition is of interest as this combination was effective in TOLERANCE induction [79]. This might also apply to interference in adhesion processes, such as the interaction between LFA-1 and ICAM-1, and that between CD2 and LFA-3. Unfortunately, anti-LFA-1 mAb were withdrawn from the market because of severe adverse side effects. The HUMANIZED anti-CD2 mAb siplizumab is presently in clinical trials for psoriasis [80], GVHD [81], and kidney transplantation; the CD2 blocker alefacept has been on the market for psoriasis since 2003 [82]; and clinical trials in kidney transplantation and GVHD are ongoing. With regard to the interaction between VLA-4 and VCAM-1, the HUMANIZED mAb natalizumab, on the market since 2004 for MS and Crohn's disease, is of interest. The application of this mAb is limited because of the incidence of JC virus-associated progressive multifocal leukoencephalopathy [83].

On the side of the ANTIGEN-PRESENTING CELL (APC), mAb to ligands of T cell COSTIMULATORY MOLECULES, like CD80 and CD86, have been used successfully in transplantation models, but the respective mAb did not enter clinical development. The primatized anti-CD80 mAb galiximab is presently in development for B lymphoid malignancies [84]. Blocking CD40 is especially attractive since CD40 is expressed not only on dendritic APC, but also on MACROPHAGES and B LYMPHOCYTES. However, there is also some expression on epithelial cells during inflammatory processes, although the non-stimulatory mAb described above [59] appears not to bind to epithelium-expressed CD40 [85]. A derivative of this Ab is now in early clinical development for PSORIATIC ARTHRITIS.

Intracellular targets, other than those described above, are found in the signaling pathway emanating from the TCR and include, among others, T cell-selective molecules such as protein tyrosine kinases of the src family ($p56^{lck}$ and $p59^{fyn}$) and ZAP-70 [86]. The pivotal and selective role of ZAP-70 for T cell activation is documented by the severe combined immunodeficiency phenotype of humans who lack functional ZAP-70. However, a clinical development program for a specific ZAP-70 inhibitor has not been established thus far. All these kinases are potential targets for the development of new immunosuppressants, but a major challenge is to identify inhibitors with high selectivity that do not inhibit other kinases of critical importance in other cell types. In addition, there are a number of molecules that do not exhibit catalytic function but act as specific adaptor molecules by mediating the interaction between different components of signal transduction pathways. Generally, these proteins contain domains that are important for protein-protein interactions such as SH2/SH3 domains. These interactions are being studied as potential drug targets as well.

CYTOKINES and cytokine RECEPTORS are suitable targets for therapeutic intervention in immune responses and inflammatory processes. Anti-TNF-α mAb (Tab. 1) were the first BIOLOGICALS to become available for patients with RA, INFLIXIMAB being the first in 1988, and these reagents are currently broadly used [87]. Annual sales of the mAb INFLIXIMAB, ADALIMUMAB, certolizumab, and the fusion protein ETANERCEPT approached US$18 billlion in 2009 (only in one other class of BIOLOGICALS, namely major cancer mAb, were similarly high sales achieved). Another anti-inflammatory product on the market for RA is the IL-1-R antagonist ANAKINRA [88]. As far as CYTOKINES involved in immune responses are concerned, the paradigm of CYTOKINES associated with Th1/Th2 cells is relevant, because transplant rejection represents primarily a TH1 RESPONSE, whereas transplantation TOLERANCE may be favored by Th2 cells. CYTOKINES promoting Th2 responses (e.g., IL-4) or blockade of CYTOKINES promoting Th1 responses (e.g., IL-12) might induce a beneficial deviation of the IMMUNE SYSTEM towards a Th2 response. CYTOKINES such as TGF-β, IL-10 and IL-35 might be attractive targets as these CYTOKINES inhibit the TH1 RESPONSE [89].

Tolerance

The final goal in IMMUNOSUPPRESSION in transplantation and AUTOIMMUNITY is the induction of TOLERANCE,

often referred to as the 'Holy Grail'. Immune TOLERANCE, or unresponsiveness to transplant rejection, which translates to a status of ALLOGRAFT function and life support without the necessity of chronic IMMUNOSUPPRESSION, can be relatively easily induced in rodent transplantation models, for instance in rats by using a short 2–3-week course of IMMUNOSUPPRESSION. This is not the case in humans or non-human primate models. In the clinic, blood transfusions (either from unrelated donors or donor-specific transfusions) have given a first indication that the IMMUNE SYSTEM can be 'modulated' to reduce reactivity towards the grafted organ. At the Ig/Ab level, the mechanism of action of IVIG in modulating immune reactions and inflammatory processes has been outlined above.

At the cellular level, much attention is currently being given to so-called Treg LYMPHOCYTES, T cells expressing CD4, CD25 and the intracellular transcription factor FoxP3. These cells and their function have been studied extensively in mouse models, and have been associated with disturbed immune regulation in AUTOIMMUNE DISEASES in humans. In animal models of allogeneic hematopoetic STEM CELL TRANSPLANTATION as treatment for leukemia, a potent suppressive effect of Treg cells has been demonstrated on immune EFFECTOR CELLS reactive to host ANTIGENS, resulting in prevention of GVHD while preserving the graft-*versus*-leukemia effect [90]. This was followed by clinical trials in patients with hematological cancers subjected to ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION. In the transplant setting, Treg LYMPHOCYTES could provide a potential cell therapy to induce TOLERANCE [91]. Amongst others, this suggestion is based on observations of increased numbers of Treg LYMPHOCYTES in the circulation of patients with 'operational' TOLERANCE. Also, it has been documented that lymphocyte subpopulations differ in SENSITIVITY to immunosuppressants. For instance, Treg LYMPHOCYTES appear to be more resistant to mTOR inhibition than other T LYMPHOCYTE subpopulations, such as effector T LYMPHOCYTES [92]. This phenomenon could contribute to a beneficial role of mTOR INHIBITORS, in combination with costimulatory blockade, to induce 'operational TOLERANCE'.

In clinical transplantation, it is now a well-known fact that patients can reduce immunosuppressive medication late after transplantation. Complete withdrawal of IMMUNOSUPPRESSION is possible, in particular in patients after liver transplantation. About 20–25% of liver transplant patients develop a state of 'operational TOLERANCE' which appears to be unrelated to the immune status of donor and recipient, age, pretransplant liver disease status, length of time for which immune suppression is given, and whether or not the patient shows chimerism with donor-derived cells in the circulation [93]. It has been proposed that this type of operational TOLERANCE occurs especially for liver grafts because of the size of the transplant ('high-dose TOLERANCE'). Other factors related to the size and composition of the graft include the production of MHC ANTIGENS by the graft, induction of microchimerism by STEM CELLS transplanted together with the liver, and an overload with donor-derived passenger LEUKOCYTES. Interestingly, the status of operational TOLERANCE appears to be related to increased proportions of Treg cells in the circulation and in the graft, as well as to increased proportions of a subpopulation of T LYMPHOCYTES, namely Tγδ cells expressing Vδ1 gene segments [94]. This subset normally occurs in the intraepithelial T cell population in the intestine, and has been associated with substantial production of IL-10 promoting Th2 immune responses.

Immunosuppressive protocols and other strategies to promote TOLERANCE induction in transplant patients are currently under investigation [79, 95]. Blocking costimulatory pathways is one of these strategies. Also, the balance between strength of INDUCTION TREATMENT and maintenance of IMMUNOSUPPRESSION is a focus of investigation, as exemplified by the anti-CD52 mAb alemtuzumab mentioned earlier. The administration of this mAb can result in a substantially lower dose of immunosuppressants during maintenance while sustaining an adequate immunosuppressed state [50, 51]. An even more stringent approach was followed using an anti-CD3 mAb conjugated to a toxin. A chemical conjugate between an anti-CD3 mAb and a mutated diphtheria toxin yielded long-term ALLOGRAFT survival of rhesus monkey kidney or islet allografts when given in the peritransplant period [96]. This result is ascribed to the severe T cell-depleting effect of the immunotoxin, which not only included T cell depletion from the blood circulation as observed with an anti-CD3 Ab,

but also depletion from LYMPHOID ORGANS. These promising results have not been pursued in patients, and the immunotoxin has subsequently been evaluated successfully in exploratory trials in cutaneous T cell lymphoma [97].

Operational TOLERANCE, as described above, is also called peripheral TOLERANCE to differentiate it from so-called central (or deletional) TOLERANCE, i.e. TOLERANCE at the level of precursors of LYMPHOCYTES. The procedure to achieve central TOLERANCE comprises the elimination of the functioning IMMUNE SYSTEM, including STEM CELLS in the BONE MARROW, by HIGH-DOSE CHEMOTHERAPY and/or whole body and thymic irradiation, followed by ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION, treatment with ATG and a short course of CsA. In this situation, a condition of mixed hematopoietic chimerism is created, in which the individual accepts solid organ transplants from the BONE MARROW donor without the need of additional IMMUNOSUPPRESSION [98]. This procedure has been shown to be effective in large animal transplantation models, including pigs and non-human primates. Subsequently, success was achieved in exploratory trials in patients with multiple myeloma and end-stage kidney disease requiring a kidney graft [99]. A major drawback of this approach is the very stringent conditioning regimen which limited the treatment of patients (multiple myeloma) that are eligible for ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION and in addition suffer from end-stage kidney disease. In search for a more subtle approach, a low intensity conditioning regimen, comprising total lymphoid irradiation and ATG given the day after transplantation, proved to be effective in mediating TOLERANCE in a recipient of a HLA-matched kidney who had no malignancy [100]. Apparently, the matching of major HLA types, i.e., transplantation across minor MHC ANTIGENS, was necessary to achieve TOLERANCE using this protocol. Success has also been noted for living donor kidney transplantation in which there was HLA mismatch for only one haplotype, using a non-myeloablative conditioning regimen [101]. This regimen comprised CY, the HUMANIZED anti-CD2 mAb siplizumab, CsA and thymic irradiation. Subsequent maintenance IMMUNOSUPPRESSION with CsA was discontinued at about 1 year after transplantation, and in four out of five patients long-lasting TOLERANCE was obtained. Besides stable kidney graft function, no donor-reactive T cell response was detectable in laboratory assays.

Summary

A broad armamentarium of immunosuppressives that are efficacious in prevention or treatment of rejection of a transplant in patients with end-stage organ failure is currently on the market or in advanced clinical development. These drugs can roughly be divided into low molecular weight XENOBIOTICS, orally active drugs produced by micro-organisms or chemical synthesis, and BIOLOGICALS, (monoclonal) Ab or rDNA fusion proteins. Most XENOBIOTICS work intracellularly and affect different pathways in lymphocyte activation and/or proliferation. Since such pathways are not truly selective for LYMPHOCYTES, most of these drugs show inherent adverse side effects and generally have a low therapeutic window. However, the availability of novel agents with a broader therapeutic window and refinements in combination treatment have greatly added to improved tolerability (synergy in IMMUNOSUPPRESSION but not adverse side effects). Broadly acting CYTOTOXIC DRUGS with severe side effects are gradually being replaced by compounds with a more selective action towards lymphoid cells, and corticosteroid-sparing regimens are applied to reduce the adverse side effects of corticosteroids. Most BIOLOGICALS work by binding to cell surface molecules, resulting in inactivation or depletion of the TARGET cells. Progress in this area has not only resulted in improved IMMUNOSUPPRESSION, but also in potential approaches to induce a status of unresponsiveness, i.e., TOLERANCE to the transplant. This is achieved either by efficient depletion of reactive cells or by inhibition of second signals in COSTIMULATION blockade of T cell activation.

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Relevant websites

http://clinicaltrials.gov/. This is a registry of federally and privately supported clinical trials conducted in the United States and around the world.

http://www.immunetolerance.org/. The Immune Tolerance Network is an international clinical research consortium founded by the National Institutes of Health, whose mission is to accelerate the clinical development of immune tolerance therapies through a unique development model

http://www.tts.org/. The Transplantation Society will provide the focus for global leadership in transplantation, by development of the science and clinical practice, scientific communication, continuing education, and guidance on the ethical practice

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Corticosteroids

Ian M. Adcock, Kazuhiro Ito and Paul Kirkham

Introduction

Cortisol secretion increases in response to any stress in the body, whether physical (such as illness, trauma, surgery, or temperature extremes) or psychological. However, this hormone is more than a simple marker of stress levels – it is necessary for the correct functioning of almost every part of the body. Excesses or deficiencies of this crucial hormone also lead to various physical symptoms and disease states [1]. Although cortisol is not essential for life *per se*, it helps an organism to cope more efficiently with its environment with particular metabolic actions on glucose production and protein and fat catabolism. Nevertheless, loss or profound diminishment of cortisol secretion leads to a state of abnormal metabolism and an inability to deal with stressors, which, if untreated, may be fatal [2].

The body's level of cortisol in the bloodstream displays a DIURNAL VARIATION, that is, normal concentrations of cortisol vary throughout a 24-hour period. Cortisol levels in normal individuals are highest in the early morning at around 8 a.m. and are lowest just after midnight. This early morning dip in cortisol level often corresponds to increased symptoms of inflammatory diseases in man [3]. Overlaid upon this DIURNAL VARIATION is the pulsatile nature of cortisol release under the control of local and central 'clocks' [4]. By mimicking this pulsatile cortisol release it is hoped to reduce the detrimental side effects of exogenous steroids while enhancing their anti-inflammatory properties [5].

Increased levels of corticosteroids serve as potent suppressors of the immune and INFLAMMATORY SYSTEMS. This is particularly evident when they are administered at pharmacological doses, but also is important in normal immune responses. As a consequence, corticosteroids are widely used as drugs to treat inflammatory conditions such as arthritis, ASTHMA or dermatitis, and as adjunct therapy for conditions such as AUTOIMMUNE DISEASES (see chapters C4 and C15). SYNTHETIC corticosteroids may also be used in organ transplantation to reduce the chance of rejection (see chapter C12). Thus, although the early effect of cortisol is to stimulate the IMMUNE SYSTEM, cortisol and SYNTHETIC corticosteroids predominantly repress the inflammatory response by decreasing the activity and production of immunomodulatory and inflammatory cells [3].

The usefulness of corticosteroids in treating inflammatory diseases was exemplified by the early work of Kendall and Hench [6]. In a classic experiment, 100 mg cortisone was injected into the muscle of a patient (Mrs G.) suffering from chronic RHEUMATOID ARTHRITIS on 21 September 1948. Seven days later the patient was able to walk to the shops for the first time in years. Kendall and Hench were awarded the Nobel Prize for this work in 1950, and it represented a new approach to therapy with NATURAL hormones by utilizing pharmacological, rather than physiological, doses.

There are five main aspects of INFLAMMATION: (i) the release of inflammatory mediators such as HISTAMINE, PROSTAGLANDINS, LEUKOTRIENES, CYTOKINES and CHEMOKINES; (ii) increased blood flow in the inflamed area (erythema) caused by some of the released factors; (iii) leakage of plasma from the vasculature into the damaged area (edema) due to increased capillary permeability; (iv) cellular infiltration signaled by chemoattractants; and (v) repair processes such as fibrosis. Corticosteroids can modify all of these processes [3].

INFLAMMATION is a central feature of many chronic diseases including dermatitis, RHEUMATOID ARTHRITIS (RA), inflammatory bowel disease (IBD, Crohn's disease and ulcerative colitis), SYSTEMIC LUPUS ERYTHEMATOSUS (SLE), ASTHMA and chronic obstructive pulmonary disease (COPD). The specific character-

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istics of the inflammatory response in each disease and the site of INFLAMMATION differ but both involve the recruitment and activation of inflammatory cells and changes in the structural cells of the lung. All are characterized by an increased expression of CYTOKINES, CHEMOKINES, GROWTH FACTORS, enzymes, RECEPTORS and ADHESION MOLECULES. The increased expression of these proteins is the result of enhanced gene transcription since many of the genes are not expressed in normal cells but are induced in a cell-specific manner during the inflammatory process [7].

Chemical structures

Corticosteroids are 21-carbon steroid hormones (Fig. 1) composed of four rings [8, 9]. The basic

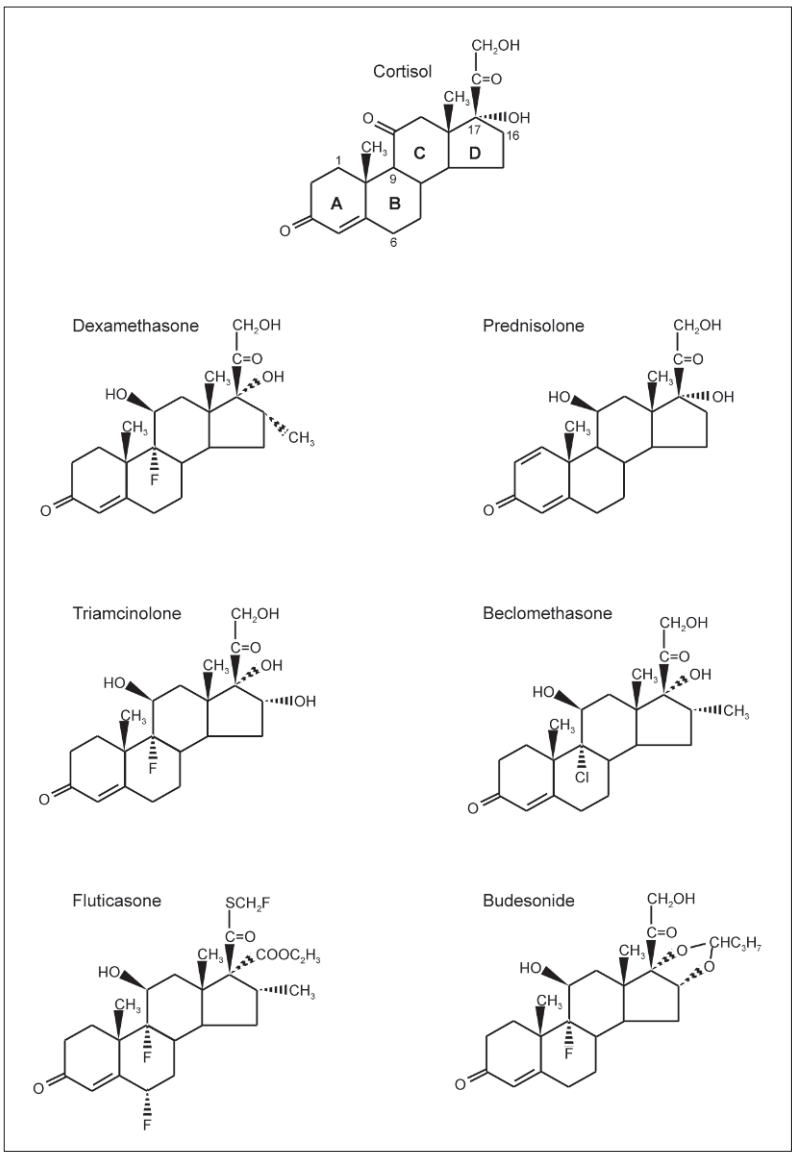

FIGURE 1.
Structural modifications of cortisol exhibited by the clinically used corticosteroids dexamethasone, prednisolone, triamcinolone, beclomethasone, fluticasone and budesonide.

structure of the A ring is a 1α, 2β-half-chair, whatever the substitutions. Rings B and C are semi-rigid chairs with minimal structural influence by substituent groups. In contrast, the shape of the D ring depends on the nature and environment of the substituent groups. Modern TOPICAL corticosteroids are based on the cortisol structure with modification to enhance the anti-inflammatory effects such as insertion of a C=C double bond at C1,C2 or by the introduction of 6α-fluoro, 6α-methyl, 9α-fluoro and/or further substitutions with α-hydroxyl, α-methyl or β-methyl at the 16 position, for example in dexamethasone (Fig. 1). Lipophilic substituents, such as 16α-, 17α-acetals, 17α-esters or 21α-esters, attached to the D-ring were found to further enhance RECEPTOR AFFINITY, prolong local TOPICAL deposition and enhance hepatic metabolism and are exemplified by the structures of budesonide and fluticasone, two of the most commonly used TOPICAL corticosteroids. The LIGAND binding cleft of the glucocorticoid RECEPTOR (GR) has been crystallized in the presence of a number of ligands and indicates a pocket that sits under the C17 residue of the steroid backbone [10]. Filling of this pocket on the floor of the binding cleft is thought to affect binding characteristics such as AFFINITY, duration and side effect profile. Using this structural knowledge has allowed the production of non-steroidal GR agonists (SEGRAs), which fill the GR LIGAND cleft spatially and have many classical GR activities but can avoid the side effects associated with the steroid backbone such as association with other steroid RECEPTORS [11]. However, the exact structural and lipophilic requirements to optimize corticosteroid pharmacokinetics, tissue retention and longevity of action are still unclear and corticosteroids with improved clinical characteristics are likely to be synthesized as our knowledge in this area increases.

Modes of action

Classically, corticosteroids exert their effects by binding to a single 777 amino acid RECEPTOR (GR) that is localized to the cytoplasm of TARGET cells. GRs are expressed in almost all cell types and their density varies from 200 to 30 000 per cell [12] with an AFFINITY for cortisol of ~30 nM, which falls within the normal range for plasma concentrations of free hormone. The GR has several functional domains (Fig. 2). The corticosteroid LIGAND-binding domain (LBD) is sited at the C terminus of the molecule and is separated from the DNA-binding domain (DBD) by a hinge region. There is an N-terminal TRANSACTIVATION DOMAIN, which is involved in activation of genes once binding to DNA has occurred. This region may also be involved in binding to other transcription factors. The inactive GR is part of a large protein complex (~300 kDa) that includes two subunits of the heat SHOCK protein hsp90, which blocks the nuclear localization of GR, and one molecule of the immunophilin p59 [12].

Corticosteroids are thought to diffuse freely from the circulation into cells across the cell membrane and bind to cytoplasmic GR (Fig. 3). Once the corticosteroid binds to GR, hsp90 dissociates allowing the nuclear localization of the activated GR-corticosteroid complex and its binding to DNA [12]. GR combines with another GR to form a dimer at consensus DNA sites, termed GLUCOCORTICOID RESPONSE ELEMENTS (GREs), in the regulating regions of corticosteroid-responsive genes. This interaction allows GR to associate with a complex of DNA-PROTEIN MODIFYING AND REMODELLING PROTEINS including steroid RECEPTOR coactivator-1 (SRC-1) and CBP, which produce a DNA-protein structure that allows enhanced gene transcription [12]. The particular LIGAND and the number of GREs and their position relative to the transcriptional start site may be important determinants of the magnitude of the transcriptional response to corticosteroids [12]. Indeed, recent evidence has suggested that the specific sequence of a GRE and its local DNA environment can act like a GR LIGAND, which endows gene- or GRE-specific characteristics to aspects of GR function [13, 14].

The GR complex can regulate gene products in at least four other ways. Firstly, GR acting as a monomer can bind directly, or indirectly, with the transcription factors ACTIVATOR PROTEIN-1 (AP-1) and NUCLEAR FACTOR κB (NF-κB), which are up-regulated during INFLAMMATION, thereby inhibiting the pro-inflammatory effects of a variety of CYTOKINES [12]. There is much dispute about the exact nature of this interaction between activated GR and NF-κB, for example, and

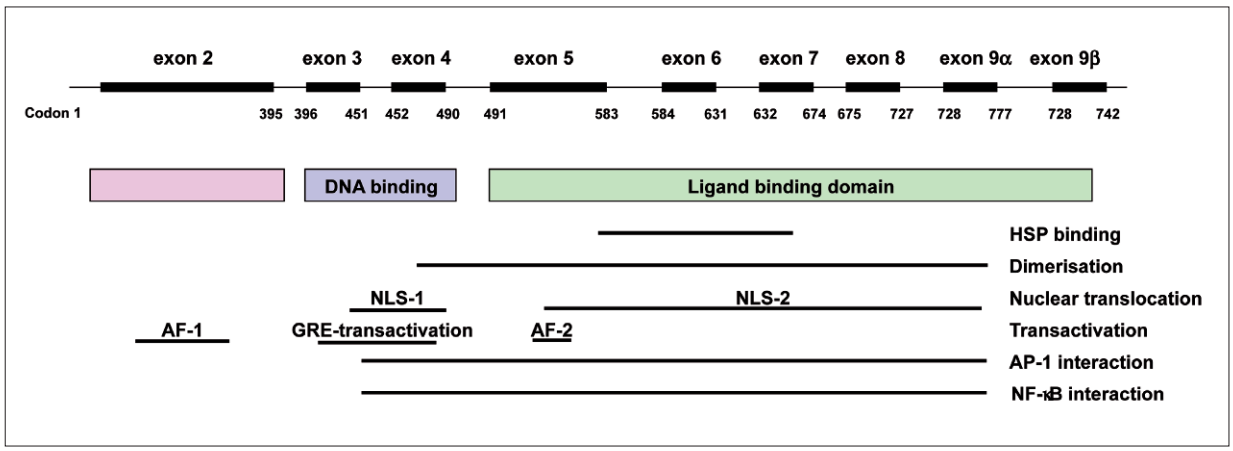

Figure 2. Modular structure of the corticosteroid receptor (glucocorticoid receptor, GR)
The coding region of GR results from splicing together of exons 2–9 of the GR gene. The GRβ isoform of GR results from the use of the short 9β exon, which removes the ligand binding domain seen in GRα. The modular design of GR enables distinct regions of the protein to function in isolation as ligand-binding domains, dimerization domains, nuclear localization domains, transactivation and transrepression (AP-1 and NF-κB interacting) domains. NLS: nuclear localization signal; AF-1/2: activating factor 1/2 and GRE: glucocorticoid response element (composed of two palindromic half sites (AGAACA) separated by three nucleotides).

the PRECISE effects are likely to be gene, cell and/or stimulus specific. Many of the proposed mechanisms reflect tethering of GR to the p65 subunit of NF-κB on κB-specific DNA binding sites in the promoter regions of inflammatory genes. Alternative mechanisms include GR recruitment of transcriptional co-repressor proteins such as HISTONE DEACETYLASE (HDAC)2 [15] or alterations in the phosphorylation status of the C-terminal repeat of RNA polymerase 2 [16]. These aspects are reviewed in depth elsewhere [17, 18].

Secondly, the GR dimer can bind to a GRE, which overlaps the DNA binding site for a pro-inflammatory transcription factor or the start site of transcription [12]. Thirdly, the GR dimer can induce the expression of the NF-κB inhibitor IκBα in certain cell types [19]. Lastly corticosteroids can increase the levels of cell ribonucleases and mRNA-destabilizing proteins, thereby reducing the levels of mRNA [20] (Fig. 3). It is likely that the altered transcription of many different genes is involved in the anti-inflammatory action of corticosteroids in ASTHMA, but the most important action of these drugs is likely to be inhibition of transcription of cytokine and chemokine genes implicated in asthmatic INFLAMMATION [12].

Evidence for this has been presented in a series of elegant experiments using mice expressing mutated GRs unable to dimerize and subsequently bind to DNA. Thus, Schutz and colleagues [21, 22] have confirmed a role for binding to DNA of GR as a dimer in the control of PRO-OPIOMELANOCORTIN (POMC) expression and T cell APOPTOSIS, but not in the modulation of inflammatory genes regulated by AP-1 or NF-κB. Finally, GR is subjected to many POST-TRANSLATIONAL MODIFICATIONS, particularly phosphorylation, acetylation and nitration, and this can have major effects on all aspects of GR function, from LIGAND binding and nuclear translocation to co-factor association and control of gene transcription [15, 17].

The traditional genomic theory of steroid action, whether through direct interaction with DNA or involving cross-talk with other transcription factors, does not fully explain the rapid effects of hormonal steroids, and it is thought that the non-genomic

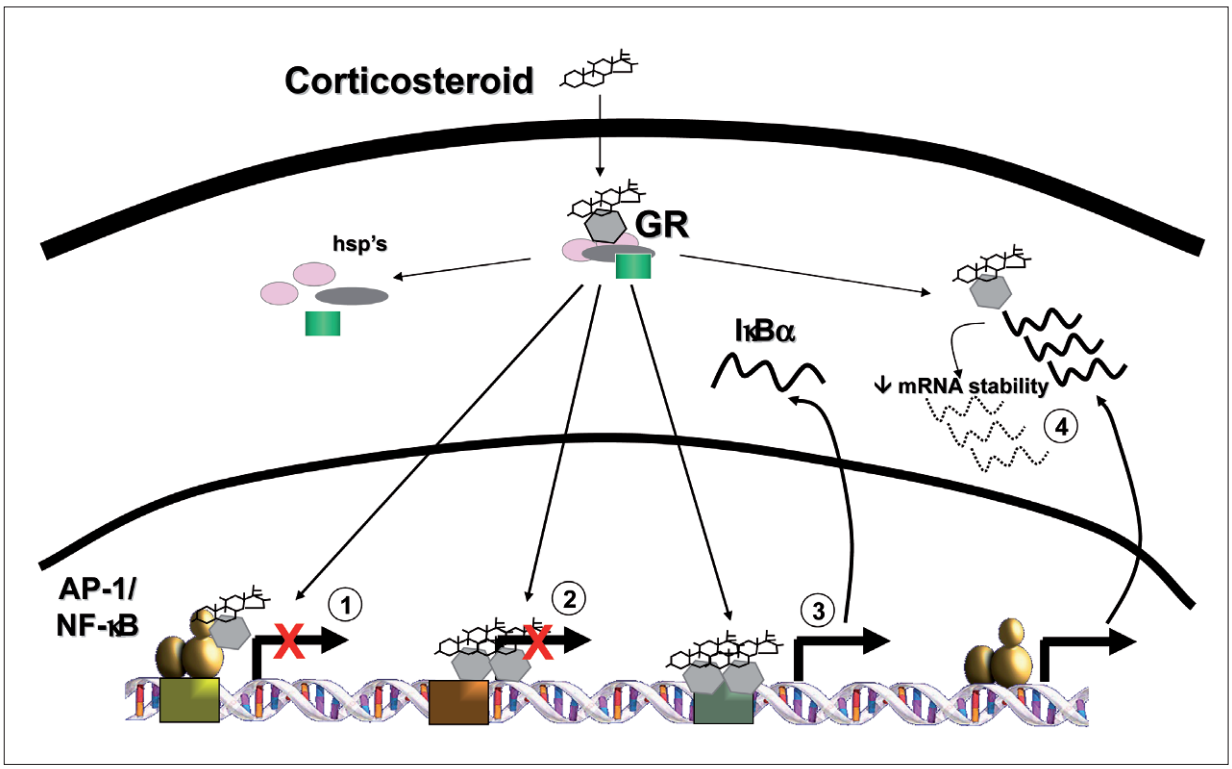

Figure 3. Mechanisms of gene repression by the corticosteroid receptor (GR)
The corticosteroid can migrate freely across the plasma membrane where it associates with the cytoplasmic corticosteroid receptor (GR). This results in activation of the GR and dissociation from the heat shock protein (hsp90) chaperone complex. Activated GR translocates to the nucleus where it can bind as a monomer either directly or indirectly with the transcription factors, activator protein-1 (AP-1) and nuclear factor κB (NF-κB), preventing their ability to switch on inflammatory gene expression ①. Secondly, the GR dimer can bind to a glucocorticoid response element (GRE) that overlaps the DNA binding site for a pro-inflammatory transcription factor or the start site of transcription thereby preventing inflammatory gene expression ②. Thirdly, the GR dimer can induce the expression of the NF-κB inhibitor IκBα ③ and fourthly corticosteroids can increase the levels of cell ribonucleases and mRNA destabilizing proteins, thereby reducing the levels of mRNA ④.

actions are mediated by a distinct membrane receptor [23]. These receptors have distinctive hormone binding properties, compared to the well-characterized cytoplasmic receptor, and are probably linked to a number of intracellular signaling pathways, acting through G protein-coupled receptors and a number of kinase pathways [23]. There are a number of reviews that provide a summary of the evidence for the rapid effects seen, which include effects on actin structures, kinase activities and transmembrane currents [24]. In addition, the classical receptor is associated with a variety of kinases and phosphatases within the inactive GR/hsp90 complex. These enzymes are released upon hormone binding and may also account for the rapid induction of tyrosine kinase activity seen in some cell types by glucocorticoids [25]. Evidence of immediate responses is also seen clinically since systemic doses of corticosteroid can lead to very rapid clinical improvement and inhibition of allergic/anaphylactic responses [24].

Pharmacological effects

Effects on inflammation

Corticosteroids are the only therapeutic agents that clearly reverse the INFLAMMATION present in chronic diseases such as dermatitis, RA, Crohn's disease, SLE, ASTHMA and COPD [3] (see chapters C4 and C15). TOPICAL and systemic corticosteroids have similar pharmacological effects, with differences related to the dose delivered to the TARGET organ and to the enhanced effect of systemic corticosteroids on the mobilization and recruitment of inflammatory cells from the blood and BONE MARROW [26].

RA is a chronic systemic inflammatory disease of undetermined etiology involving primarily the synovial membranes and articular structures of multiple joints. The disease is characterized by the INFLAMMATION of the membrane lining the joint, which causes pain, stiffness, warmth, redness and swelling and finally, later in disease, joint destruction [27]. The inflamed joint lining the synovium, can invade and damage bone and cartilage. Inflammatory cells release enzymes that may digest bone and cartilage. The involved joint can lose its shape and alignment, resulting in pain and loss of movement. Corticosteroids are effective in reducing joint pain, stiffness and swelling, and the release of inflammatory mediators and tissue-digesting enzymes [28]. Evidence for slowing of the radiographic progression of disease with corticosteroids has been available for more than 45 years [28]. Despite this, the use of corticosteroids in RA remains controversial, primarily because of possible undesirable side effects [28].

In general, in all chronic inflammatory and immune diseases, corticosteroids cause a marked reduction in the number and activation of infiltrating cells, including MAST CELLS, MACROPHAGES, T LYMPHOCYTES, and EOSINOPHILS, in the inflamed tissue [3]. Furthermore, TOPICAL and oral corticosteroids can have affects on tissue resident cells and in ASTHMA, for example, can reverse the shedding of epithelial cells, goblet-cell hyperplasia and basement-membrane thickening that is characteristically seen in biopsy specimens of bronchial epithelium from patients [3] (Fig. 4).

Cellular effects

Corticosteroids may have direct inhibitory effects on many of the cells involved in INFLAMMATION, including MACROPHAGES, T LYMPHOCYTES, EOSINOPHILS, MAST CELLS, smooth muscle, endothelial and epithelial cells, resulting in reduced mediator synthesis and release [3]. In general, corticosteroids substantially reduce mast cell/eosinophil/lymphocyte driven processes, while leaving unaltered, or even augmenting, neutrophil-mediated processes [29]. For example, corticosteroids may enhance neutrophil function as a result of increased LEUKOTRIENE and superoxide production, in addition to inhibiting their APOPTOSIS [29]. Corticosteroids also decrease platelet CLEARANCE by the reticuloendothelial (mononuclear phagocyte) system, decrease ANTIBODY production, and by stabilizing capillaries decrease bleeding in patients with immune thrombocytopenic purpura (ITP) [30]. Furthermore, in autoimmune hepatitis, prednisolone prevents T cell recognition of AUTOANTIGENS, intrahepatic recruitment and proliferation of LYMPHOCYTES, IgG production and may enhance suppressor T cell function in these patients [31].

In allergic diseases, corticosteroids also reduce the number of MAST CELLS within the inflamed tissue; however, they do not appear to inhibit mediator release from these cells [3]. Treatment with TOPICAL corticosteroids also reduces the number of activated T LYMPHOCYTES ($CD25^+$ and $HLA\text{-}DR^+$) in bronchoalveolar lavage (BAL) fluid and peripheral blood from asthmatic patients [32]. Corticosteroids are particularly effective against eosinophilic INFLAMMATION, possibly as a result of decreasing eosinophil survival by stimulating APOPTOSIS [33]. Interestingly, some patients with difficult-to-control ASTHMA may develop exacerbations despite treatment with TOPICAL corticosteroids, and these often appear to have a T cell-, rather than an eosinophil-, dependent inflammatory mechanism [34, 35].

In addition to their suppressive effects on inflammatory cells, corticosteroids may also inhibit plasma exudation in most tissues and mucus secretion in inflamed airways [29]. There is an increase in vascularity in chronic inflammatory diseases [36] and high doses of TOPICAL systemic corticosteroids may reduce both this [36] and the increased blood flow present at sites of INFLAMMATION [37].

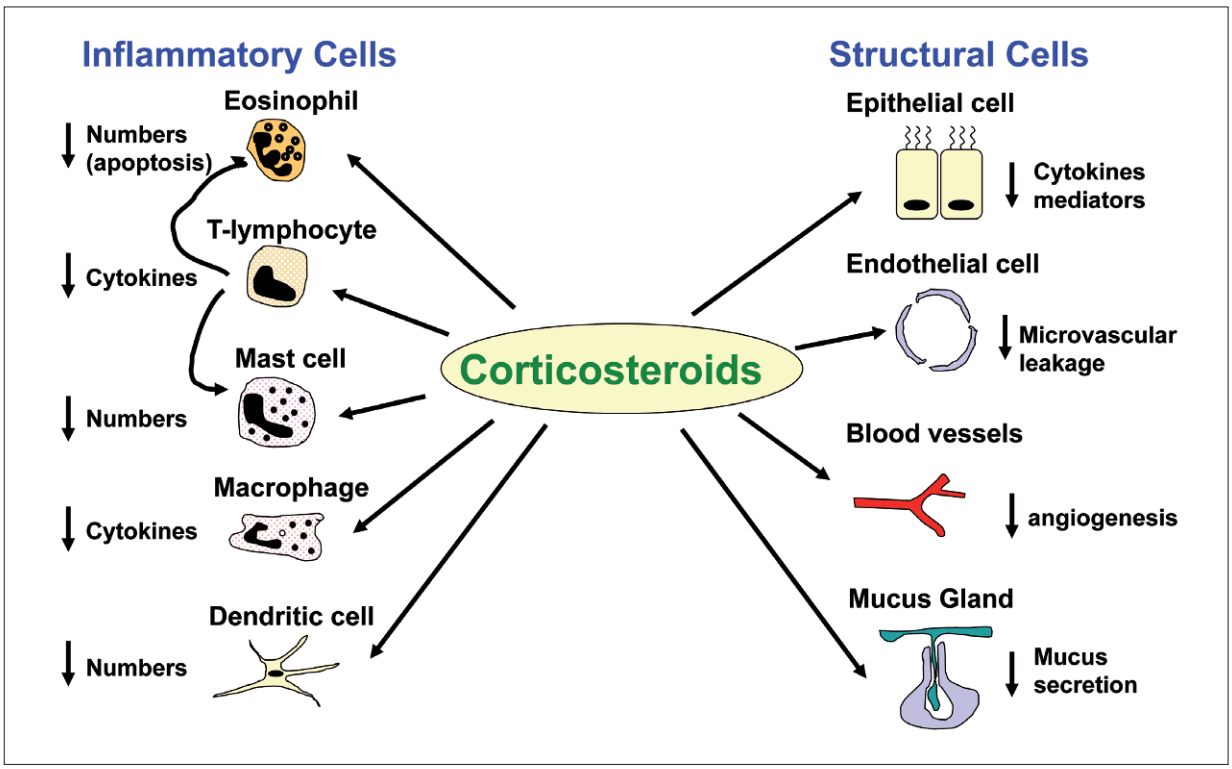

FIGURE 4

Corticosteroids act on most infiltrating and resident inflammatory cells to suppress inflammation. The activity (T lymphocytes and macrophages) and/or number of infiltrating cells (eosinophils, T lymphocytes, macrophages, mast cells and dendritic cells) are decreased by corticosteroids. Corticosteroids also have a suppressive effect on resident tissue cells and can reduce mediator release and adhesion molecule expression on epithelial and endothelial cells, microvascular leakage from blood vessels, angiogenesis and both the numbers of mucus glands and release of mucus from these glands.

Mediator effects

Corticosteroids block the generation of most pro-INFLAMMATORY CYTOKINES and CHEMOKINES, including INTERLEUKIN (IL)-1β, IL-4, IL-5, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α), RANTES and macrophage inflammatory protein-1α (MIP-1α) [29]. Despite the wide pleiotropy (multiple actions) and redundancy that exists in the cytokine and chemokine families, and the subsequent inability to ascribe PRECISE roles to most of these molecules in inflammatory disease pathogenesis, it is clear that these proteins are important mediators in chronic INFLAMMATION (see chapter A5). The recent development of blocking ANTIBODIES and inhibitors to TNF-α has provided evidence that, in RA at least, this molecule plays a key driving role in INFLAMMATION [38, 39] (see chapter C15). This does not appear to be the case with all inflammatory diseases, however [38–40]. Interestingly, corticosteroids can also enhance the expression of key anti-inflammatory molecules, such as IL-10 and IL-1ra, in some systems but again not all [3, 26]. For example, corticosteroids increase the production of IL-10, but not IL-1ra, at sites of INFLAMMATION in ASTHMA [41, 42].

Arachidonic acid metabolism *via* 5-LIPOXYGENASE gives rise to a group of biologically active lipids known as LEUKOTRIENES: LEUKOTRIENE B_4, which is a potent activator of leukocyte CHEMOTAXIS, and cysteinyl LEUKOTRIENES (LEUKOTRIENE C_4, D_4 and E_4), which account for the spasmogenic activity previously described as slow-reacting substance of ANAPHYLAXIS (see chapter A7). LEUKOTRIENES, particularly cysteinyl LEUKOTRIENES, are thought to play a key role in some chronic and acute inflammatory diseases, but do not appear to be major targets for corticosteroids [43]. Thus, dexamethasone is ineffective in inhibiting LTB_4/LTC_4 release from many cells and tissues [43]. Furthermore, systemic treatment with corticosteroids has no inhibitory effect on LEUKOTRIENE release by whole blood LEUKOCYTES, purified NEUTROPHILS, or MONOCYTES [43] and even increases LEUKOTRIENE biosynthesis by blood NEUTROPHILS *ex vivo* [44]. Interestingly, corticosteroids *in vitro* accelerate LTC_4 catabolism by inducing the activity of a LTC_4-degrading enzyme, γ-glutamyl transpeptidase-related enzyme (γ-GTPRE), in transformed human bronchial epithelial cells [45].

The INFLAMMATION in COPD [29] and in a high number of patients with IBD [46, 47] is scarcely suppressed by TOPICAL or oral corticosteroids, even at very high doses. Potential reasons for the failure of corticosteroids to function effectively in reducing INFLAMMATION in IBD and COPD include the fact that both diseases have a high oxidative load and oxidative stress may reduce CORTICOSTEROID RECEPTOR (GR) nuclear translocation [29,46,47], with reduced GRα expression or altered GR-co-factor interactions within the nucleus [29]. Interestingly, cigarette smoke contains 10^{17} oxidant particles per puff and asthmatic subjects who smoke have a reduced responsiveness to both TOPICAL and oral corticosteroids [48].

Pharmacokinetics

The pharmacokinetics of many corticosteroids has been well described. In general, plasma concentrations of corticosteroids vary considerably (up to tenfold) after oral administration of the same dose by normal volunteers and patients [49]. Many compounds have plasma elimination half lives of 2–4 hours, whereas the biological half lives range from 18 to 36 hours [50]. The pharmacokinetic properties of TOPICAL drugs depend upon a combination of tissue deposition/targeting, RECEPTOR binding, volume of distribution, tissue retention, and lipid conjugation. In addition, to achieve a good THERAPEUTIC INDEX, drugs need to possess a low oral BIOAVAILABILITY and small particle size, rapid metabolism, high CLEARANCE, high plasma protein binding and a low systemic half-life. Furthermore, an ideal compound would be inactive at sites distal to the TARGET organ [51,52].

There are two main methods of reducing the systemic activity of TOPICAL corticosteroids: (i) reducing gastrointestinal BIOAVAILABILITY, and (ii) prolonging TISSUE RESIDENCY. For example, oral administration of ileal-release budesonide capsules for the treatment of Crohn's disease gives similar levels of systemic exposure to active drug, BIOAVAILABILITY and cortisol suppression in adults and children as seen with prednisolone, but importantly, no clinically relevant adverse side effects were reported [53]. In ASTHMA, changing the inhaler device can decrease oral delivery and subsequently gastrointestinal availability and enhance lung deposition by altering the particle size. Alternatively, for IBD the corticosteroid can be altered to reduce gastrointestinal absorption and/or enhance first-pass hepatic metabolism. Prolonged retention in the tissue can be achieved by increasing lipophilicity, as with fluticasone propionate (FP) and mometasone furoate, or by forming soluble intracellular esters, as with budesonide and ciclesonide [51, 52].

Lipophilicity generally correlates well with absorption characteristics. For example, fluticasone has high lipophilicity and binding AFFINITY for the GR, resulting in a high volume of distribution and long plasma half-life. However, the systemic side effects of fluticasone that arise from systemic absorption are limited due to its almost complete first-pass metabolism and, in the case of enteric delivery, low absorption from the gastrointestinal tract. In general, for TOPICAL corticosteroids, treatment EFFICACY and side effects are directly related to tissue dose. This may also vary depending upon disease severity in the case of fluticasone, but not, apparently, with

budesonide [51, 52]. The pharmacokinetic profile of TOPICAL corticosteroids, therefore, varies with the individual drug, the delivery mechanism and patient profile.

Clinical indications

Pharmacological control of INFLAMMATION may be obtained in most patients with varying doses of oral or TOPICAL corticosteroids, depending upon the disease severity [3,54]. In most patients, adequate doses of TOPICAL corticosteroids allow systemic administration to be reduced or withdrawn completely [3]. As such, corticosteroids are standard therapy for disorders such as RA, ASTHMA, connective tissue diseases, vasculitis, allergic reactions, IBD, psoriasis and eczema. However, when administered over a long period of time at reasonably high concentrations, their beneficial effects are overshadowed by a number of side effects including OSTEOPOROSIS, skin atrophy and diabetes [54].

Overall, the duration, dosage and dosing regime, the particular corticosteroid used and its mode of administration along with the patient's individual susceptibility, appears to determine the incidence of adverse events [54, 55]. Prolonged TOPICAL application is also a high risk factor, whereas total dose is of secondary importance. Not surprisingly, side effects are much more severe with systemic corticosteroid use, although even TOPICAL application can induce both local and systemic side effects [56]. The presence of these side effects is the limiting factor for therapy and has driven research and development of new compounds [54, 57]. The newer potent TOPICAL steroids, such as mometasone and fluticasone, seem to carry a lower risk for adrenal suppression than older drugs [58].

Prednisone and prednisolone are highly effective in inducing remission of Crohn's disease [59, 60]. Crohn's disease causes INFLAMMATION predominantly in the ileum, although other parts of the gut may be involved. Crypt INFLAMMATION (cryptitis) and abscesses progress to focal ulcers, which, in some cases, regress and in others, the inflammatory process evolves with influx and proliferation of MACROPHAGES and other inflammatory cells, occasionally forming non-caseating granulomas with multinucleated giant cells. Transmural INFLAMMATION, deep ulceration, edema, muscular proliferation, and fibrosis cause deep sinus tracts and fistulas, mesenteric abscesses, and obstruction, which are the major local complications. As with other diseases, however, because they have systemic effects, the use of corticosteroids has been associated with an increased incidence of adverse events [60].

Once it was demonstrated that corticosteroids were effective in inducing remission of Crohn's disease, oral drug delivery systems were developed to provide the EFFICACY of a corticosteroid without the associated toxicity. The choice of drug is based on the physicochemical and pharmacological properties of the corticosteroid, although this varies greatly between individual subjects [49]. However, TOPICAL corticosteroids are still mostly unproven clinically, possibly due to a lack of appropriate delivery systems [59, 60].

For example, local therapy of distal colitis has proved effective using retention enemas of hydrocortisone and betamethasone [59] and animal models have shown the feasibility of colonic delivery to increase local tissue concentrations and reduce adverse side effects of corticosteroids [61]. Budesonide is the most commonly used TOPICAL corticosteroid in IBD, and was initially used because of its extensive first-pass hepatic metabolism, which reduces oral BIOAVAILABILITY (10–15%) [62]. Budesonide also possesses a high rate of metabolism compared to other corticosteroids, such as beclomethasone and triamcinolone. Enteric-coated sustained release pellets of budesonide (10 mg) are as effective as 40 mg prednisolone in controlling IBD endoscopic scores but do not affect plasma cortisol levels [60]. In an ongoing study of more than 4000 Crohn's disease patients treated with doses of up to 21 mg/day budesonide, some for more than 5 years, serious adverse events were experienced by only 3% of patients; most of these were gastrointestinal and unrelated to treatment [63, 64].

Immunosuppressive agents such as 6-mercaptopurine and AZATHIOPRINE are also used to treat Crohn's disease. These drugs may cause side effects like nausea, vomiting, and diarrhea and may lower

the patient's resistance to infection. However, when patients are treated with a combination of corticosteroids and immunosuppressive drugs, the immunosuppressives are CORTICOSTEROID SPARING [60].

Corticosteroids are also the most widely used therapy in dermatology. Initially treatment involved hydrocortisone, but great advances were seen following the introduction of halogenated compounds, such as triamcinolone and the more potent drugs such as budesonide and fluticasone [54]. This inevitably led to over-prescription with adverse events becoming increasingly apparent. "Steroid phobia" is still a considerable concern [54,58] and over 70% of dermatology patients in the UK express worries about TOPICAL corticosteroid use, 24% being non-compliant as a result. Skin thinning was the major fear (35%) with 10% being concerned about systemic absorption and effects on growth and development [58].

Other chronic immune and inflammatory diseases, such as SLE, ITP (an autoimmune bleeding disorder due to lack of platelet formation) and autoimmune hepatitis are also treated predominantly with systemic corticosteroids. Corticosteroids are generally taken orally, the dose varying with disease severity and organ involvement. Intramuscular methylprednisolone or hydrocortisone may be used during a mild flare characterized by fatigue or arthralgia in SLE for example, but more aggressive therapy is often required [65]. Despite initially good response rates to corticosteroid therapy ranging from 65 to 78% for ITP for example, this soon drops to <20% in patients with chronic disease [66]. It is unclear whether this reflects a reduced EFFICACY of the drugs or a lack of PATIENT COMPLIANCE. Evidence that patients may respond to intravenous dexamethasone [30] suggests that the latter may be a problem, possibly due to the presence, or fear, of adverse events.

In ASTHMA, corticosteroids consistently lessen airway hyperesponsiveness and the maximal response to a number of spasmogens and irritants [3, 67, 68]. Interestingly, the reduction in airway hyperresponsiveness may not be maximal until treatment has been given for several months. The magnitude of the reduction varies, and airway responsiveness can remain abnormal [3]. When corticosteroid therapy is discontinued, airway responsiveness usually returns to pretreatment levels [69].

Side effects

All currently available TOPICAL corticosteroids are absorbed into the systemic circulation and, therefore, inevitably have some systemic effect, although this is considerably less than those seen with oral corticosteroids (Tab. 1). The occurrence and severity of the side effects seen depend upon the duration of use, dosage, dosing regime and specific drug used, along with individual patient variability [54]. However, the highest risk factor appears to be prolonged use. Side effects of TOPICAL steroids include glaucoma, cataracts, tissue atrophy and delayed wound healing, while at high doses there is an increased risk of infection, adrenal suppression and OSTEOPOROSIS. The growth retardation seen with oral corticosteroids does not appear to be a problem with modern TOPICAL corticosteroids, although there may be an initial reduction in growth velocity on starting therapy. Side effects of oral corticosteroids include skin and muscle atrophy, delayed wound healing and increased risk of infection, OSTEOPOROSIS and bone necrosis, glaucoma and cataracts, behavioral changes, hypertension, peptic ulcers and gastrointestinal bleeding and Cushing' syndrome and diabetes [54]. Interestingly, it appears that early skin atrophy induced by corticosteroid therapy is reversible, whereas major atrophy leading to striae formation is not [58].

These side effects often occur together and this is exemplified by Cushing's syndrome (hypercortisolism), signs and symptoms, including elevated blood pressure, development of diabetes, pink-to-purple stretch marks on the abdominal skin, fatigue, depression, moodiness, and accentuated fatty tissue on the face and upper back (Buffalo hump) [1, 54]. Women with Cushing's syndrome often have irregular menstrual periods and develop new facial hair growth. Men may show a decrease in sex drive. Taken together, these side effects seriously limit the value of corticosteroids in severe INFLAMMATION where the risk/benefit ratio is compromised. This has driven the need to develop novel agents with the anti-inflammatory capacity of corticosteroids but with reduced side effects.

While the major anti-inflammatory effects of corticosteroids are almost certainly due to TRANSREPRESSION,

Table 1. Tissue/organ-specific side effects of topical and systemic corticosteroids

Cardiovascular system	Hypertension Dyslipidemia Thrombosis Vasculitis
CNS	Disturbances in mood, behavior, memory and cognition "Steroid psychosis", steroid dependence Cerebral atrophy
Endocrine system, metabolism, electrolytes	Cushing's syndrome Diabetes mellitus Adrenal atrophy Growth retardation Hypogonadism, delayed puberty Increased sodium retention and potassium excretion
Eye	Glaucoma Cataract
Gastrointestinal	Peptic ulcer Gastrointestinal bleeding Pancreatitis
Immune system	Increased risk of infection Re-activation of latent viruses
Skeleton and muscle	Muscle atrophy/myopathy Osteoporosis Bone necrosis
Skin	Atrophy, striae, distension Delayed wound healing Steroid acne, perioral dermatitis Erythema, teleangiectasia, petechia, hypertrichosis

the underlying molecular mechanisms for the side effects of corticosteroids are complex and not fully understood [54]. Certain side effects such as diabetes and glaucoma are due to TRANSACTIVATION events, whereas others are due to TRANSREPRESSION (hypothalamic-pituitary-axis, HPA, suppression). In addition, the PRECISE molecular events underlying corticosteroid induction of OSTEOPOROSIS is unclear but probably requires both gene induction and gene repression [54].

Despite this uncertainty, there has been a search for "dissociated" corticosteroids that selectively transrepress without significant TRANSACTIVATION, thus potentially reducing the risk of systemic side effects. Several non-steroidal SEGRA have recently been reported that show dissociated properties in human cells and are now in clinical development, showing good separation between TRANSREPRESSION and TRANSACTIVATION actions in the skin [12, 57, 70]. This suggests that the development of corticosteroids and SEGRA with a greater margin of safety is possible and may even lead to the development of oral compounds that have reduced adverse effects. Furthermore, the newer TOPICAL corticosteroids used today, such as fluticasone, mometasone and budesonide, appear to have more potent transrepressing than transactivating effects, which may account, at least in part, for their selection as potent anti-

inflammatory agents [12]. These new potent corticosteroids are particularly effective as TOPICAL agents and their use has overtaken that of oral/systemic corticosteroids for many diseases. For example, coated enteric slow-release budesonide capsules are equally effective as prednisolone in Crohn's disease without the associated reduction in plasma cortisol seen with prednisolone [59, 71]. Similar results have been achieved with fluticasone albeit in fewer well-controlled studies [72].

An alternative approach to obtain safer drugs is the use of soft drugs, such as ciclesonide, which are only activated at the site of INFLAMMATION. Ciclesonide is a novel inhaled corticosteroid for the treatment of ASTHMA. Ciclesonide itself is inactive and needs to be cleaved by lung-specific esterases to bind to the CORTICOSTEROID RECEPTOR [73]. According to data from healthy volunteers and ASTHMA patients, ciclesonide affects serum cortisol levels significantly less compared to beclomethasone dipropionate [73] or fluticasone propionate [74], indicating that ciclesonide might have less systemic effects and hence a superior safety profile.

Other approaches to the production of 'safer' corticosteroids are the use of alternative agents that TARGET other aspects of the inflammatory response and therefore act as steroid-sparing agents. These classes of drug include kinase inhibitors, immunomodulatory agents such as CICLOSPORIN and long-acting β-agonists (LABA) [7].

Summary

Corticosteroids are the most effective therapy for chronic immune and inflammatory diseases in current use. Despite their success over the past 50 years, and especially since the advent of new potent halogenated compounds, worries about the detrimental side effects of systemic corticosteroids have limited their effectiveness in severe disease. This has resulted in the increasing use of TOPICAL corticosteroids targeted to the site of INFLAMMATION rather than systemic administration. Improvements in risk/benefit ratios are likely to occur, as greater understanding of the role of chemical substitution of the SYNTHETIC corticosteroids becomes clear, and more potent tissue selective drugs are developed. Drugs that TARGET distinct aspects of corticosteroid function, switching on or off genes, are also under development and, along with non-steroidal agents that TARGET different aspects of the inflammatory response, are likely to lead to safer drugs with a much reduced side-effect profile. However, until these become widely available, current systemic and TOPICAL corticosteroids are likely to remain the major treatment for most inflammatory diseases.

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A comprehensive information resource for patients with Crohn's disease and ulcerative colitis: *http://ibd.patientcommunity.com/links.cfm?parentcat_id=109&cat_id=109*

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Non-steroidal anti-inflammatory drugs

C14

Regina M. Botting and Jack H. Botting

Introduction

Throughout history humans have experimented with herbal remedies to alleviate the symptoms of diseases. The active principles of some of these remedies are of proven value and have become established in modern therapeutics. None, however, has been more widely accepted nor as universally practised as the use of the extracts of certain plants for the treatment of the various symptoms of inflammatory conditions such as pain, swelling and fever.

The Egyptian Ebers papyrus records that 3500 years ago extracts of the dried leaves of myrtle applied to the abdomen and back were beneficial for rheumatic pains from the womb. A thousand years later, no less an authority than Hippocrates recommended the juice of willow bark to reduce fever and alleviate the pain of childbirth. Similar curative effects were attributed to decoctions from Salix and Spirœa species by early inhabitants of North America and South Africa.

The beneficial effects of willow bark were placed on a more scientific basis by the observations of a country parson, the Reverend Edward Stone of Chipping Norton in Oxfordshire. Stone gathered a pound of willow bark, dried it over a baker's oven and ground it to a fine powder. Doses of 1 dram (1.8 g) were found to be successful in 50 patients with fever [1]. The restriction of the availability of willow bark due to the use of willows for manufacture of wickerware resulted in herbalists cultivating meadowsweet (*Spiraea ulmaria*) to provide treatment for the ague and similar conditions.

By the middle of the nineteenth century advances in chemistry established that the common constituent of the plant extracts that reduced fever and inflammatory pain was salicylate. Salicylic acid was synthesized in Germany in 1860, and its ready availability led to its widespread use in fever, rheumatism and as an external antiseptic. The value of salicylate as a medicine was limited by its unpleasant taste and tendency to produce nausea. In an attempt to make a more palatable preparation, Felix Hoffman, a chemist working for the Bayer Company, synthesised acetylsalicylate or aspirin. Bayer's Research Director, Dr Heinrich Dreser, tested the effects of aspirin in animals and in the clinic and, in 1899, introduced it as an antipyretic, anti-inflammatory and analgesic drug [2]. Aspirin has become perhaps the most widely used of all drugs, and its value as an antipyretic and for the pain of rheumatoid- and osteoarthritis is well accepted. Sporadic reports of gastrotoxicity produced by aspirin were substantiated in 1938 by endoscopic studies, which clearly demonstrated that aspirin produced erosions and even frank ulceration of the gastric mucosa [3].

The realisation that this valuable medicine could produce a serious and sometimes fatal gastrotoxicity stimulated a search for compounds with antipyretic, analgesic and anti-inflammatory actions without gastrotoxicity. Many compounds with differing chemical structures were produced and marketed from 1940 onwards, but all possessed the gastrotoxicity to some degree. As a group, these drugs were designated "non-steroid anti-inflammatory drugs" (NSAIDs) (see Appendix C14/I).

Mode of action of NSAIDs

The fact that many compounds of diverse chemical structure (see Appendix C14/II) not only possessed the same therapeutic actions but also shared identical toxic side effects raised the intriguing possibility

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that a single biochemical action was responsible for all of the various actions of the NSAIDs. Many biochemical effects of NSAIDs were demonstrated: inhibition of dehydrogenases, aminotransferases, decarboxylases and several key enzymes involved in protein and RNA biosynthesis, as well as many others. However, there was no obvious correlation between these effects and the therapeutic and toxic actions of NSAIDs, and they were achieved only with concentrations well above those found in human plasma after therapy.

The enigma of the mechanism of action of NSAIDs was ultimately resolved by the elegantly simple pharmacological experiments of Vane (1971) [4], who showed that aspirin and some other NSAIDs inhibited, in a dose-dependent manner, the synthesis of the highly active lipid mediators, PROSTAGLANDINS (PGs), from guinea pig lung homogenates. Vane hypothesised that both the therapeutic and side actions of NSAIDs were due to inhibition of PG synthesis. PGs are formed from arachidonic acid mobilised from membrane phospholipids by a phospholipase enzyme. Arachidonic acid is acted upon by the microsomal enzyme CYCLOOXYGENASE (COX) to form the cyclic endoperoxides PGG_2 and PGH_2. These unstable endoperoxides are then isomerised, enzymatically or non-enzymatically, into various prostanoids such as THROMBOXANE A_2 (TXA_2), PROSTACYCLIN (PGI_2) and PGD_2, PGE_2 and $PGF_{2\alpha}$ (see Fig. 1). Aspirin and other NSAIDs inhibit the COX enzyme, thus preventing the formation of the endoperoxide precursors of the various PGs.

PROSTAGLANDINS were known to be pyrogenic and were shown to be present in cerebrospinal fluid during fever. Similarly, PGE_2 and PGI_2 are vasodilator and present at inflammatory foci (such as the synovial fluid of arthritic joints), suggesting their involvement in the swelling typical of inflammatory conditions (see chapter A7). The analgesic action of NSAIDs was initially less easy to explain, since PGs, unlike other mediators of INFLAMMATION such as BRADYKININ, were not pain-producing substances. However, they were subsequently shown to greatly potentiate the pain induced by other mediators, that is, they manifested a "hyperalgesic" effect. Clearly, the three therapeutic actions of NSAIDs could be explained by inhibition of PG synthesis.

That the common side effects of NSAIDs, such as gastrotoxicity and nephrotoxicity, were also due to inhibition of PG synthesis was apparent when PGs were shown to be cytoprotective on the gastric mucosa, and could maintain renal blood flow when renal circulation was compromised. The decreased platelet reactivity observed, particularly after aspirin, was explained when the prostanoid TXA_2 was shown to be a potent inducer of platelet aggregation [5].

COX-1 and COX-2

Implicit in the establishment of the mechanism of action of NSAIDs was the assumption that since all the pharmacological actions depended on the inhibition of COX, it would be impossible to separate the therapeutic effects from the toxic actions of these agents. However, there were some minor inconsistencies in this theory. Epidemiological and experimental studies showed that the severity of gastric toxic effects of different NSAIDs varied when the drugs were used at comparable anti-inflammatory doses. Ibuprofen, for example causes less damage to the stomach than ketoprofen [6]. Similarly, the NSAID nimesulide was effective in models of inflammatory disease such as the carrageenan-injected rat paw, yet was poorly active in inhibiting PG synthesis in conventional systems [7].

The existence of multiple COX enzymes was a likely explanation for these inconsistencies; over 30 years ago Flower and Vane had suggested that paracetamol, which lacks anti-inflammatory actions, exerted its antipyretic effect by inhibition of a distinct isoform of COX in brain tissue [8]. More recently a number of workers showed that PG synthesis could be up-regulated in inflammatory conditions by induction of synthesis of more COX enzyme. For example, Needleman and colleagues [9] showed, using a model of the inflammatory condition of hydronephrosis in perfused rabbit kidney, that there was a marked increase in PGE_2 release following injection of BRADYKININ. This increased release could be prevented by prior treatment with the protein synthesis inhibitors cycloheximide or actinomycin D. Of significance, aspirin easily inhibited the release of PG

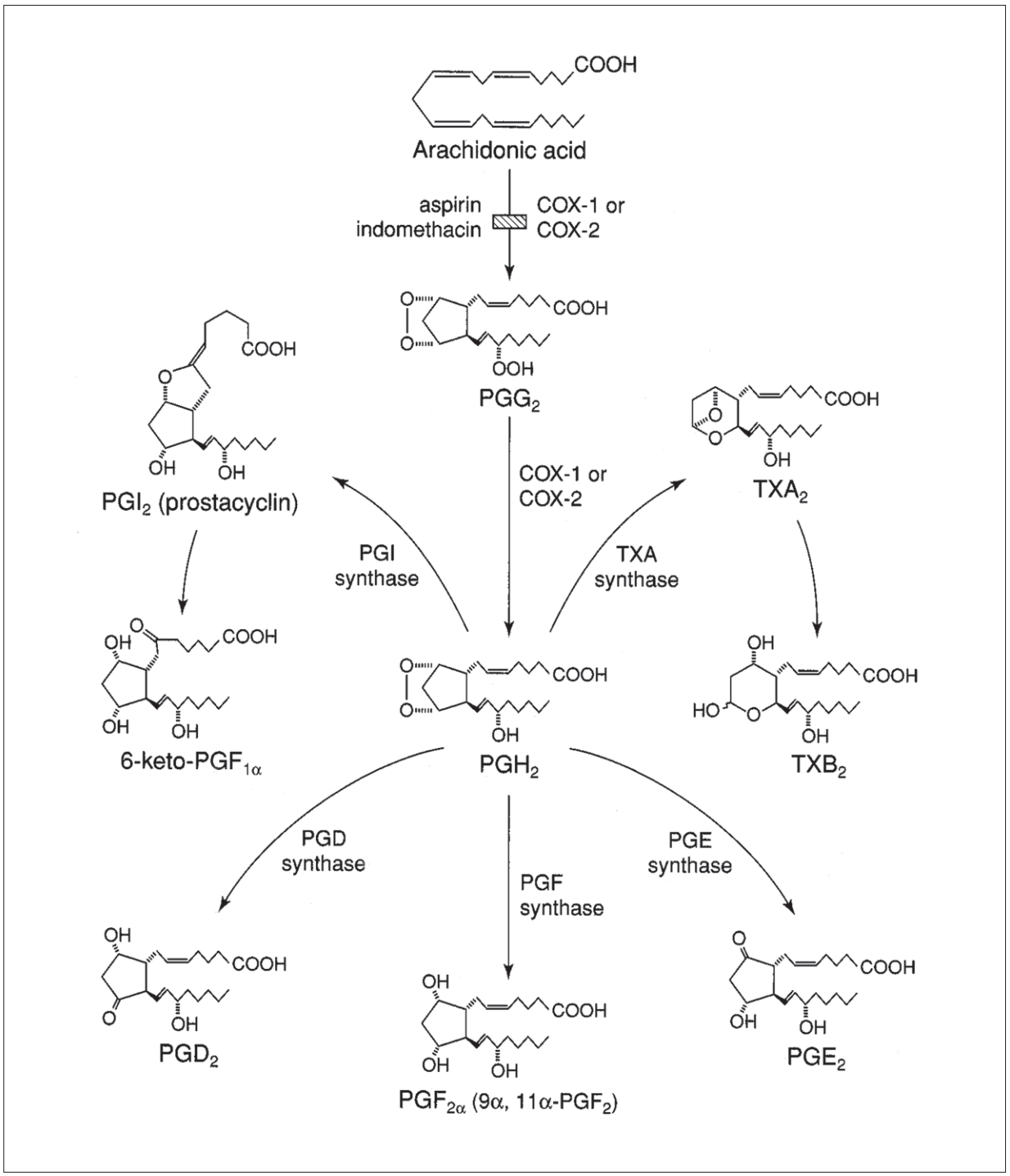

Figure 1. The arachidonic acid cascade

during the early stages of perfusion, but became less effective as the experiment progressed. Needleman concluded that the increase in PG production in this model of INFLAMMATION was secondary to increased synthesis of COX enzyme [9].

Similar results were obtained from experiments using isolated cells stimulated with various INFLAMMATORY CYTOKINES or GROWTH FACTORS. Human umbilical vein endothelial cells so treated increased production of PGI_2, together with an increase in COX protein and mRNA [10], effects reduced by treatment with actinomycin D.

Needleman and his group [11–13] extended their earlier observations by demonstrating increased PG synthesis in human MONOCYTES *in vitro* and in mouse MACROPHAGES *in vivo* after activation with LPS. The increase in PG release was inhibited by prior treatment with dexamethasone, yet the basal level of COX activity was not affected. Needleman concluded that there may be various "pools" of COX within cells, one constitutive, another inducible, being up-regulated by inflammatory stimuli [13]. The existence of such an inducible COX enzyme was firmly established through the discovery by Simmons and his colleagues at Brigham Young University, of a novel PG synthase, encoded by an entirely different gene, induced in chick embryo fibroblasts by v-src, serum or phorbol esters [14, 15]. Subsequently, Herschman and his colleagues [16] found a similar gene in the mouse, as did Simmons et al. [17] who, by cross HYBRIDISATION, cloned and characterised the murine homologue of the v-src-inducible COX and showed the mRNA to be induced in an immediate early fashion by various mutagens. The deduced protein structure of the inducible enzyme was found to be 60% homologous to constitutive COX, and the two enzymes were designated COX-1 (constitutive) and COX-2 (inducible).

Although COX-1 is constitutive in most cells, very little COX-2 is present in resting cells. However, it is powerfully induced in fibroblasts, endothelial cells and vascular smooth muscle by mitogens or some CYTOKINES, and in monocytic cells by LPS. These observations have led to the general hypothesis that the constitutive COX-1 is a "housekeeping" enzyme involved in maintaining normal physiological processes. Thus, COX-1 is responsible for synthesising TXA_2 involved in platelet aggregation, PGE_2 and PGI_2 to protect the gastric mucosa and PGI_2 in the vascular endothelium to maintain dilatation of blood vessels and to inhibit formation of inappropriate platelet thrombi (although recent work suggests that formation of PGI_2 from endothelial cells may be due to COX-2 constantly induced in the cells by shear stress [18]).

The inducible COX-2, however, is believed to be primarily responsible for the production of PGs in pathological processes, for example the swelling and hyperalgesia associated with inflammatory disease (see Fig. 2). Certainly, increased COX-2 expression, which parallels increase in PGE_2 synthesis, is demonstrable in models of inflammatory disease such as the murine air pouch, whereas COX-1 expression is unchanged [19]. Increased expression of COX-2 has also been demonstrated in synovial tissue taken from patients with RHEUMATOID ARTHRITIS, compared to tissues from patients with osteoarthritis or no arthritic pathology [20].

The implication that the COX-2 isoform was responsible for the synthesis of PGs at pathological foci and COX-1 for the synthesis of the beneficial "housekeeping" PGs, raised the exciting possibility that selective inhibitors of COX-2 would exert the therapeutic actions of NSAIDs without, or with less of, the previously regarded inevitable side effect of gastrotoxicity. This prompted many workers to undertake studies to establish the relative activity of established NSAIDs on COX-1 and COX-2 by comparison of the concentrations of NSAID necessary to inhibit the activity of each COX by 50% (IC_{50}). Although simple in concept, these measurements of selectivity were problematic in practice. Differences in source of enzyme, time of incubation, method of induction of COX-2 and protein concentration between various laboratories resulted in a variation in ratios and even differences in IC_{50} values. However, the human whole blood assay developed by Patrignani et al. [21] and subsequently modified by Warner [6], provided a reliable method with similar incubation times and appropriate human TARGET cells, and which performed in the presence of plasma proteins. In this method, the action of the NSAID on COX-1 is determined by the inhibition of production of the TXA_2 metabolite (TXB_2), whereas the effect on COX-2 is assessed by the inhibition of PGE_2 formation

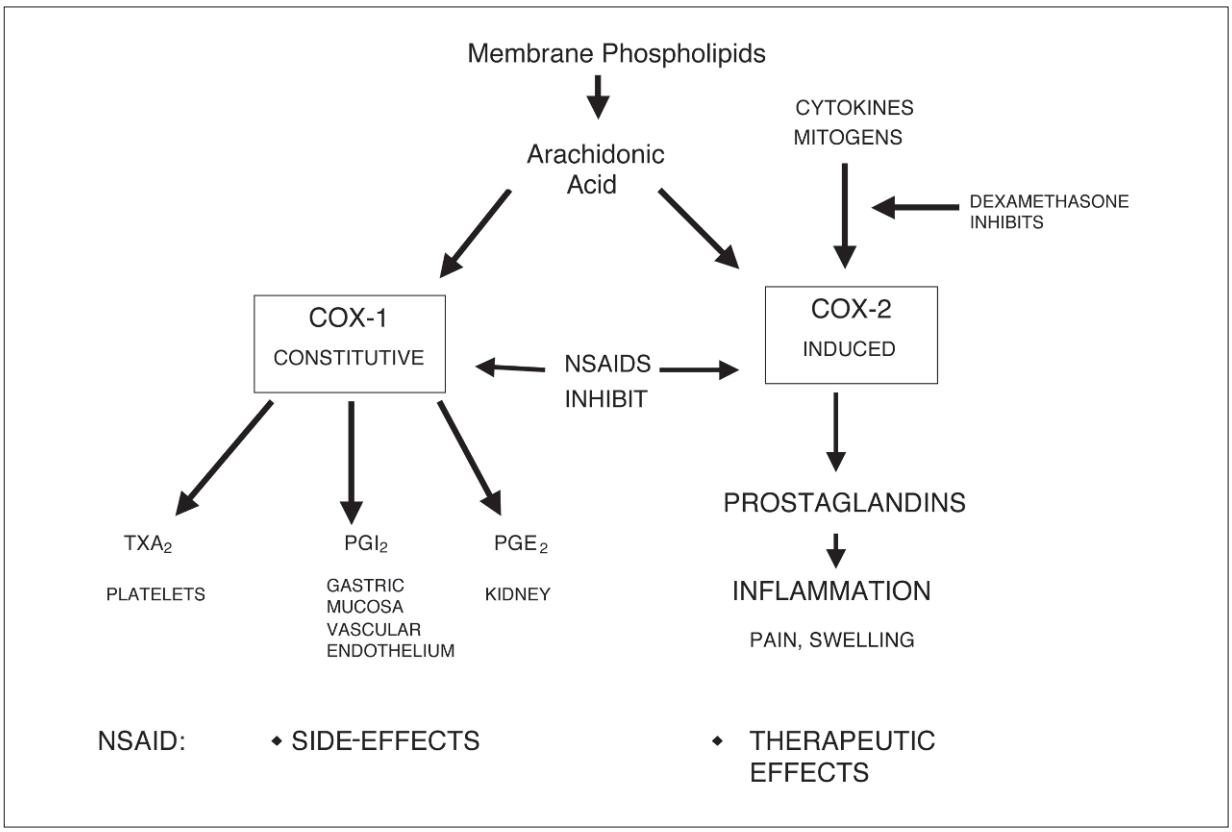

Figure 2

The side effects of NSAIDs are caused by inhibition of the constitutive enzyme COX-1, which synthesises prostaglandins (PGs) that serve essential physiological functions such as causing appropriate platelet aggregation, protection of the gastric mucosa, inhibition of thrombogenesis and maintenance of renal function. The therapeutic effects of NSAIDs are due to inhibition of COX-2, an enzyme induced by various factors released by bacteria, the vascular endothelium or other cells involved in the inflammatory response.

from previously activated human A549 cells added to the whole blood. This method was reproducible and the selectivity of established NSAIDs towards inhibition of COX-2, rather than COX-1 was shown, in large-scale clinical trials and post-marketing surveillance studies, to be accompanied by relatively low gastrotoxicity. Thus, nimesulide, for example, which is approximately 20 times as active on COX-2 compared to COX-1, has low gastrotoxicity, whereas ketorolac, which is many hundred times more selective for COX-1, has a relative risk of 25, compared to a value of 4–5 for general NSAID use.

Structural basis for COX-2 selectivity

The three-dimensional structures of both COX-1 [22] and COX-2 [23] have been determined and are remarkably similar. Each enzyme is composed of three distinct folding units: an epidermal growth factor-like domain, a membrane-binding section and a C-terminal enzymatic domain containing both the COX and peroxidase active sites. Through the membrane-binding domain, the enzymes integrate into a single leaflet of the membrane lipid bilayer,

thus positioning the COX site to allow access of the arachidonic acid SUBSTRATE, which is mobilised from adjacent membrane phospholipids.

Whereas NSAIDs generally act competitively to prevent access of arachidonic acid to the active site, aspirin acts in a unique manner to acetylate a serine residue at position 530 in COX-1, or the serine at an analogous position (516) in COX-2. Although the active sites of COX-1 and COX-2 are similar, there are differences that have been utilised by medicinal chemists to synthesise molecules that have a selective action on COX-2. Of crucial significance is position 523, which in COX-2 is valine and in COX-1 isoleucine. This difference of a single methyl group on an amino acid is sufficient to allow access of a potential inhibitor to a side pocket in the COX-2 enzymatic domain and, in addition, proximity to an arginine residue at position 513 (histidine in COX-1), which provides hydrogen bonding for an inhibitor of a structure, enabling it to extend into the side pocket [24] (Fig. 3).

A second significant difference between COX-2 and COX-1 is at position 503, which is the aromatic amino acid, phenylalanine in COX-1 but the relatively small, non-aromatic leucine in COX-2. This allows leucine at position 384 to reorient its methyl side chain away from the enzymatic site and thus extend the space available for a larger inhibitor molecule at the active site of the COX-2 enzyme [25].

Selective COX-2 inhibitors

Drugs specifically designed to inhibit COX-2 have entered the market and several such drugs have undergone comprehensive clinical investigation. Celecoxib is the prototype of a number of highly selective COX-2 inhibitors produced by Monsanto/Searle and is 30 times as potent on COX-2 as on COX-1. Celecoxib was shown to be an effective analgesic as assessed in patients after tooth extraction, and endoscopic studies after 3 months chronic usage showed no more gastric damage than with placebo. In a large scale clinical trial (the Celecoxib Long-term Arthritis Safety Study or CLASS, in 8000 patients) it was shown that, at 6 months, celecoxib produced less gastrotoxicity than the comparator drugs, ibuprofen and diclofenac [26]. However, in the CLASS study, extended for 12 months, celecoxib demonstrated equal gastrotoxicity to ibuprofen and diclofenac. Celecoxib is approved for the treatment of osteoarthritis and RHEUMATOID ARTHRITIS in many countries including the USA and UK.

Rofecoxib, produced by Merck, Sharp and Dohme, is 272-fold more potent for COX-2 than for COX-1 and in doses of 25–50 mg is an effective analgesic for dental pain, dysmenorrhoea and osteoarthritis. In a 6-month endoscopic study, with a dose of rofecoxib

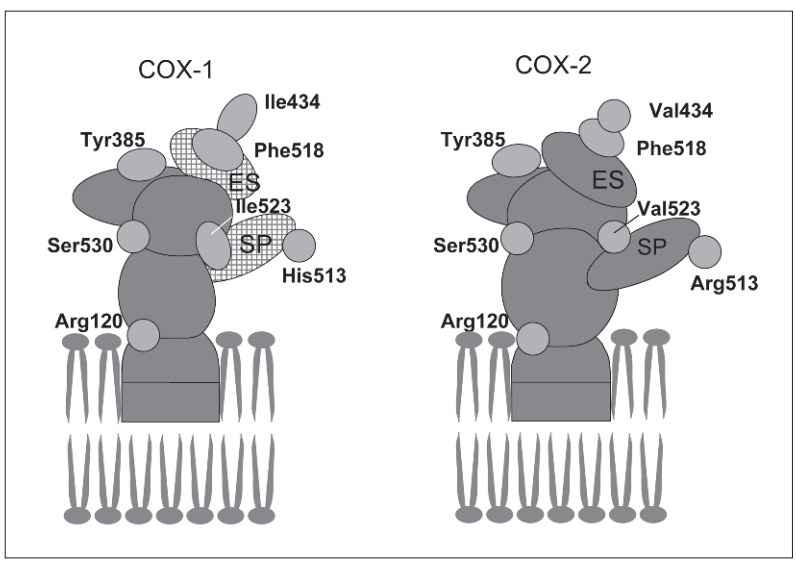

FIGURE 3. AMINO ACID SUBSTITUTIONS BETWEEN COX-1 AND COX-2
The hatched areas in COX-1 are those that are more accessible in COX-2 due to the amino acid substitutions. SP, side pocket; ES, extra space

10-fold greater than that required for an effective anti-inflammatory effect, gastric damage was no greater than that seen with placebo. In a large-scale, 9-month trial involving 8000 patients (the Vioxx Gastrointestinal Outcome Research, VIGOR), rofecoxib was compared with naproxen [27]. Rofecoxib was shown to produce substantially less gastrotoxicity than naproxen. However, in the VIGOR study the incidence of myocardial infarction in the rofecoxib group was 0.4%, compared with 0.1% in the naproxen-treated patients. In a further trial of rofecoxib for the prevention of colorectal adenomatous polyps [28], polyp numbers were reduced after 3 years, but an increased incidence of adverse cardiovascular events resulted in the premature termination of this trial. These observations eventually led to the withdrawal of rofecoxib from the market in 2004.

Monsanto/Searle's successor to celecoxib was valdecoxib with a 61-fold greater potency against COX-2 than against COX-1. The prodrug of valdecoxib, parecoxib, an injectable analgesic was marketed at the same time and is still available in Europe but not in the USA. The Federal Drug Administration rejected registration of valdecoxib and parecoxib because both drugs were shown to increase cardiovascular risk in patients undergoing cardiovascular bypass graft surgery [29, 30] and because of the possibility of serious skin reactions with valdecoxib.

Etoricoxib, Merck's successor to rofecoxib, with a selectivity for COX-2 of 344 times that for COX-1, is marketed in Europe but has failed to obtain FDA approval in the USA. It has a long half-life and can be administered as a once daily dose for the symptomatic treatment of osteoarthritis, RHEUMATOID ARTHRITIS and acute gouty arthritis, as well as for the short-term treatment of musculoskeletal pain, postoperative pain and dysmenorrhoea [31]. The gastric damage, assessed endoscopically, was less with etoricoxib than with traditional NSAIDs. In a large randomised clinical outcome study in 34 701 patients with osteoarthritis and RHEUMATOID ARTHRITIS treated for 18 months, the Multinational Etoricoxib and Diclofenac Arthritis Long-term (MEDAL) programme, the rates of thrombotic cardiovascular events were no greater for etoricoxib than for diclofenac [32].

Lumiracoxib, made by Novartis, is a weak acid and an analogue of diclofenac. It penetrates well into areas of INFLAMMATION and is 433 times more selective for COX-2 than for COX-1. It is effective in osteoarthritis, primary dysmenorrhoea, acute and postoperative pain. A large clinical trial in 18 000 osteoarthritic patients for 1 year [33, 34] demonstrated fewer gastric ulcer complications with lumiracoxib than with naproxen or ibuprofen, especially in patients not taking low-dose aspirin. There were no more cases of myocardial infarction in the lumiracoxib group of patients than in the comparator naproxen and ibuprofen groups. However, lumiracoxib was withdrawn throughout Europe by the European Medicines Agency in November 2007, because of the high risk of serious liver toxicity. It did not obtain registration in the USA.

The selective COX-2 inhibitors (COXIBs) reduce the synthesis of PGI_2 (PROSTACYCLIN) in human patients as measured by PGI_2 metabolites in the urine, thus causing unopposed overactivity of TXA_2. This results in cardiovascular side effects including myocardial infarction, hypertension and stroke. Thus, the selective COX-2 inhibitors should be prescribed in the lowest effective dose for the shortest possible time for patients who do not tolerate the administration of other NSAIDs [35, 36].

Selective COX-2 inhibitors and cancer

There is persuasive epidemiological evidence for an association of chronic use of NSAIDs with reduced incidence of gastrointestinal carcinomas [37–43]. Regular use of aspirin and other NSAIDs can halve the risk of development of colon cancer. Clinical and experimental studies have provided further evidence to justify this beneficial effect of NSAIDs; colonic tumour tissue has been shown to contain high levels of COX-2 protein but contiguous, normal tissue contains only COX-1 [44]. Patients with the inherited disorder of familial adenomatous polyposis (FAP), a condition characterised by a plethora of colonic polyps with eventual progression to tumours, manifest a marked reduction in polyp number on treatment with the NSAID sulindac [45,46]. Similarly, COX inhibitors reduce tumours in azoxymethane-induced colorectal carcinogenesis in rat [47] and

selective COX-2 inhibitors reduce tumour or polyp numbers in animal models of FAP such as minimal intestinal neoplasias (min) mice or adenomatous polyposis coli gene mutant knockout mice. A clinical trial of celecoxib in patients with FAP showed a 30% reduction in polyps and this use of the drug was approved by the Food and Drug Administration, USA [48].

More recent trials of celecoxib for the prevention of colorectal adenomatous polyps found a significant reduction in polyp numbers after 1 and 3 years of treatment with high doses [49]. However, celecoxib showed an increased risk of adverse cardiovascular events, which makes the long term use of celecoxib as a chemotherapeutic agent contraindicated, particularly in patients with cardiovascular or cerebrovascular disease. As already mentioned [28], in a parallel study of rofecoxib in the prevention of colorectal adenomatous polyps, polyp numbers were reduced after 3 years, but there was an increase in myocardial infarctions and strokes.

COX-2 is expressed in tumour tissue in many cancers other than colorectal carcinomas. Increased levels of COX-2 have been shown in human lung squamous cell and adenocarcinomas, squamous skin carcinomas and in tumours of the breast, liver, pancreas, prostate and cervical lymph nodes (for review see [50])

Other cyclooxygenases

Although the discovery of COX-2 resolved the puzzle of why some NSAIDs manifested marked gastrotoxicity, yet others, more selective for COX-2 were relatively less toxic, some enigmas remained. As mentioned above, paracetamol, like conventional NSAIDs, has marked antipyretic and analgesic activity yet lacks the typical anti-inflammatory and antiplatelet activities of this group of drugs. Twenty years ago it was suggested that paracetamol might inhibit a COX in the brain, and that various, perhaps tissue-specific, COXs may exist that are selectively inhibited by different NSAIDs [8].

Some light has recently been shed upon this field by Simmons and his colleagues who have identified a new COX-1 variant in dog and human brain that is sensitive to paracetamol and has been designated COX-3 [51]. COX-3 has the same structure as COX-1 except that the mRNA has retained intron-1, which is translated into a 30-amino acid extention to the enzyme protein. COX-3 is ectopically expressed in insect cells and found to be more sensitive to inhibition by paracetamol than either COX-1 or COX-2. Studies in transgenic mice lacking the COX-1 gene showed that the antinociceptive action of paracetamol was abolished when the COX-1 gene was absent, indicating that a COX-1 gene-derived protein (COX-3) was required for the antinociceptive activity of paracetamol [52].

Human COX-3 was found in highest concentrations in the cerebral cortex followed by the heart and skeletal muscle. The possible functions of COX-3 in organs other than the brain are as yet unknown. Simmons had previously found another COX isoform, sensitive to inhibition by paracetamol, formed by a murine macrophage cell line (J774.2) treated with high concentrations of diclofenac [53]. However, unlike COX-3, this enzyme was insensitive to aspirin, and was considered to be a variant of COX-2, since a protein immunoreactive to COX-2 was co-induced together with the CYCLOOXYGENASE activity. This COX-2 variant protein is induced by activation of peroxisome proliferator-activated RECEPTOR γ (PPARγ) and may be involved in the resolution of INFLAMMATION [54].

Summary

Since earliest times extracts of salicylate-containing plants have been used by humans to alleviate the symptoms of inflammatory conditions, i.e. INFLAMMATION, pain and fever. Eventually, salicylic acid was synthesised chemically and became widely available for the treatment of INFLAMMATION. The more palatable derivative of salicylic acid, acetylsalicylic acid, was then synthesised in 1897 and became a popular medicine under the name of 'Aspirin'. But the mechanism of action of aspirin remained unknown until 1971 when John Vane discovered that aspirin reduced the synthesis of the ubiquitous

lipid mediators, the PGs. Inhibition of the synthesis of PGs explained the anti-inflammatory, antipyretic and analgesic effects of aspirin, as release of PGs was the main cause of INFLAMMATION, fever and pain. After this discovery, many aspirin-like drugs such as ibuprofen, naproxen, piroxicam and others were developed by pharmaceutical companies and they all reduced the synthesis of PGs by inhibiting the activity of the COX enzyme that was responsible for their biosynthesis. These were collectively termed NSAIDs . However, all the NSAIDs to a lesser or greater extent shared the side action of causing bleeding and ulceration of the stomach mucosa.

A major advance in the history of the NSAIDs took place in 1991 when Daniel Simmons and his colleagues found that there were two separate genes producing distinct COX enzymes. Thus, two CYCLOOXYGENASE enzymes were identified, COX-1 and COX-2. COX-1 is the 'housekeeping' enzyme which maintains the integrity of the stomach mucosa, causes appropriate aggregation of platelets and maintains perfusion of the kidney, whereas COX-2 is an inducible enzyme produced during INFLAMMATION by inflammatory stimuli such as mitogens, CYTOKINES and inflammatory mediators.

It was soon realised that to develop NSAIDs that would reduce INFLAMMATION without damaging the stomach would be a worthwhile goal and within 10 years selective inhibitors of COX-2 were undergoing clinical trials for the treatment of arthritis. The first two selective COX-2 inhibitors, celecoxib and rofecoxib were approved by the FDA in 1999 and were prescribed for millions of arthritic patients worldwide. However, rofecoxib was withdrawn from the market in 2004 because of an increased risk of myocardial infarction. Subsequently, parecoxib, valdecoxib, etoricoxib and lumiracoxib were developed (parecoxib is the prodrug for valdecoxib). Currently, only celecoxib is available in the USA and celecoxib, parecoxib and etoricoxib can be prescribed in Europe. The newer selective COX-2 inhibitors, valdecoxib and lumiracoxib have no registration in the USA or Europe because of adverse side effects: serious skin reactions in the case of valdecoxib and fatal liver toxicity in the case of lumiracoxib.

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Disease-modifying antirheumatic drugs

Kevin D. Pile, Garry G. Graham, Stephen M. Mahler and Richard O. Day

Introduction

RHEUMATOID ARTHRITIS (RA) is the most common inflammatory rheumatic disease, with a prevalence of about 1%. Targeting the synovial membrane, cartilage and bone, untreated RA leads to joint destruction, disability and increased mortality. Although the total incidence of this disease is low, the level of ill health and economic burden is significant with the patients often partially or totally unemployed. Generally, the patients require long-term drug treatment and non-pharmacological approaches such as physiotherapy and psychosocial support. There are no reliably curative or disease-remitting therapies, although considerable gains have been made utilizing biological therapies and small molecules to TARGET specific CYTOKINES [TUMOR NECROSIS FACTOR (TNF) and INTERLEUKINS such as IL-1 and IL-6], cellular subsets (B cells, Th17 cells), and immune regulatory steps in RA.

All RA patients require treatment with a medication that in the absence of a cure, suppresses RA, a disease-modifying anti-rheumatic drug (DMARD). The most recent additions to the DMARD armamentarium are biologically engineered products, primarily immunoglobulin constructs, referred to here as biological DMARD (bDMARD). DMARD refers both to the older small molecule agents and to the developing area of small molecule inhibitors of signaling pathway proteins.

Long-term deleterious effects such as erosions of bone begin in the first 1–2 years of disease and, consequently, initiation of therapy early in RA is important. This creates a window of opportunity whereby early disease suppression prevents lasting joint damage [1]. Early treatment with DMARDs, in addition to reducing erosive progression, improves symptoms and limits disability. This approach contrasts to older regimens in which DMARD were only commenced on the appearance of bony erosions on joint X-rays. For all inflammatory arthritic diseases non-steroidal inflammatory drugs (NSAIDs) are useful symptomatically but have no clear effect on the progression of RA (see chapter C14). Many patients with RA or other inflammatory arthritic diseases are also administered intra-articular and/or low-dose oral corticosteroids. These are frequently administered as bridging therapy until the effect of systemic therapy is fully established. In some patients, they are used over a longer term to satisfactorily suppress the disease. The major challenge in therapeutics is optimizing the timing, combination and dosage of available therapies to preserve joint function, quality of life and life expectancy in RA patients.

It should be noted that the principles of the use of DMARD in RA are paralleled in their treatment of other inflammatory rheumatic diseases such as PSORIATIC ARTHRITIS and juvenile chronic arthritis.

Pathophysiology of rheumatoid arthritis

The concept of a genetic predisposition and an environmental trigger has been applied to nearly all AUTOIMMUNE DISEASES, including RA.

Genetic factors contributing substantially to the risk of developing RA are:

(i) the MAJOR HISTOCOMPATIBILITY COMPLEX genes HLA-DR1 and 4 (containing the shared epitope) which associate strongly with disease severity.

Other RA-associated loci include:

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(ii) CTLA4 (CYTOTOXIC T lymphocyte antigen 4) affecting the second costimulatory signal between antigen-presenting cells and T cells;
(iii) protein tyrosine phosphatase, non-RECEPTOR type 22 (PTPN22), which reduces T cell signaling allowing auto-reactive T cells to escape deletion;
(iv) cytokine and cytokine RECEPTOR loci (TNF, IL-1, IL-10, IL-18); and
(v) PADI 4 (peptidyl arginine deiminase type IV), which catalyzes the conversion of arginine to citrulline to generate novel ANTIGENS and has a role in the development of anti-citrullinated peptide (anti-CP) ANTIBODIES, which may precede the development of clinical synovitis by many years.

Environmental triggers for RA are postulated, although despite an intensive search for transmissible agents, no infectious cause has been proven. Smoking is the strongest environmental risk factor for RA, with a significant multiplicative interaction between heavy smoking (>10 pack years) and any HLA shared epitope, resulting in an increased risk of seropositive RA, with an odds ratio of 7.5 if a double copy of the shared epitope is present [2]. Smoking reduces NATURAL killer (NK) cells, depresses hormonal cells and CELL-MEDIATED IMMUNITY, leading to dysfunction of T cells [3], and may enhance the citrullination of self-peptides within the lung, and initiate the formation of anti-cyclic citrullinated peptide (CP) ANTIBODIES.

Pathology of RA

Despite the uncertainly relating to the initiating events, the ultimate pathology of RA, namely synovial INFLAMMATION (or synovitis), is well established. The normal synovium is a thin and delicate layer that reflects off the cartilage-periosteal border on to the underlying fibrous joint CAPSULE. Synovium is composed of cells of fibroblast and macrophage origin, and it has two major functions: the provision of oxygen and nutrients to cartilage *via* the synovial fluid, and the production of lubricants, notably hyaluronic acid, that allow the articular surfaces to glide smoothly across each another.

In RA, the synovium is transformed into a chronically inflamed tissue (Fig. 1). The normally thin synovial layer thickens dramatically due to accumulation of macrophage-like and fibroblast-like synoviocytes, and the subsynovial layer becomes edematous, hypervascular and hypercellular with the accumulation of macrophage, MAST CELLS, $CD4^+$ T cells, $CD8^+$ T cells, NATURAL killer (NK) cells, B cells and plasma cells. The increased number of cells in the synovium in RA is believed to result from recruitment of blood-derived LEUKOCYTES, as well as increased proliferation and reduced APOPTOSIS. NEUTROPHILS are abundant in rheumatoid synovial fluid but are sparse within the synovium.

In concert with this inflammatory process the inflamed synovium invades adjacent cartilage and bone. Cartilage injury caused by the generation of degradative enzymes, including matrix METALLOPROTEINASES, aggrecanases and myeloperoxidase may be a source of neoantigen and perpetuation of the INFLAMMATION (Fig. 1). Bone injury is separately mediated by a process of osteoclast activation regulated by the RECEPTOR activator of the NF-κB-RANK-LIGAND (RANKL) system, induced by macrophage and/or fibroblast production of CYTOKINES. The cells, CYTOKINES, RECEPTORS, and signaling pathways operative in these processes constitute therapeutic targets for biological therapies in RA, and many are influenced by existing DMARD.

Cytokines in RA

Cellular expansion in the synovium is accompanied by significant overproduction of CYTOKINES, which function as crucial effectors in the pathogenesis of RA, and are potential therapeutic targets [4]. Compelling evidence from *in vitro* and animal studies, as well as the effects of human treatment with cytokine antagonists, indicates that these and other CYTOKINES drive continued cell recruitment and expansion, angiogenesis, and the production of pro-inflammatory mediators, such as PROSTAGLANDINS and reactive oxygen and nitrogen species (Tab. 1). Since CYTOKINES such as TNF, IL-1 and macrophage migration inhibitory factor

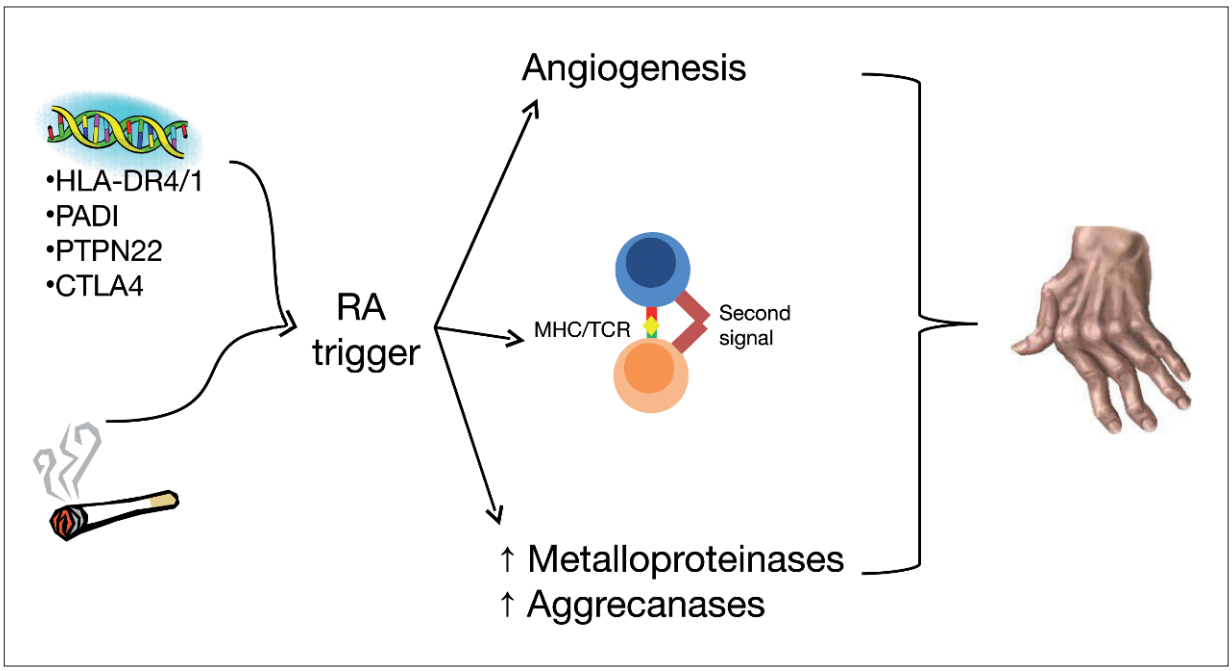

Figure 1. Schematic diagram of events in development of RA

(MIF) can induce their own synthesis and secretion, a self-perpetuating inflammatory cycle of AMPLIFICATION ensues, leading to the chronic INFLAMMATION of RA.

These advances in our understanding of the pathophysiology of RA have been mirrored in the development of targeting therapies, with the largest group to date being anti-cytokine therapy (Tab. 1). Early cytokine research identified TNF and IL-1 as important in joint INFLAMMATION and destruction. At present a range of TNF modulators such as MONOCLONAL ANTIBODIES (INFLIXIMAB, ADALIMUMAB, golimumab) PEGYLATED MONOCLONAL ANTIBODIES (see Certolizumab below) and soluble TNF RECEPTORS (ETANERCEPT) and an IL-1 RECEPTOR antagonist (ANAKINRA) are in clinical use. TNF can be produced by a variety of cells including MONOCYTES, T cells, B cells, NK cells, GRANULOCYTES, keratinocytes and smooth muscle cells, following an equally diverse range of stimuli: bacteria, viruses, trauma, irradiation, hypoxia and CYTOKINES (e.g., IL-1 and IL-17). TNF in particular has key roles within the inflammatory network as well as heading an inflammatory cascade, leading to monocyte and neutrophil activation, PROSTAGLANDIN release, APOPTOSIS of NEUTROPHILS and T cells, increased expression of ADHESION MOLECULES, increased matrix metalloproteinase production, and increased free fatty acid release from adipocytes (Tab. 1).

T cells are implicated in RA on the basis of the genetic association with the HLA shared EPITOPES and the protein tyrosine phosphatase, non-RECEPTOR type 22 (PTPN22) RECEPTOR loci, as well as their presence in the synovium and their requirement in animal models of disease. The functionality of T cells is dependent on their activation that, in contrast to many other signaling pathways, requires a dual signaling mechanism. The first is the presentation by an ANTIGEN-PRESENTING CELL of a HLA-peptide complex to the antigen-specific T cell RECEPTOR. The second or costimulatory signal is the linking of the ANTIGEN-PRESENTING CELL surface LIGAND, CD80/86, with its cognate RECEPTOR CD28 on the T cell. In the absence of COSTIMULATION, the HLA-peptide complex-T cell RECEPTOR interaction induces a state of T cell anergy in which the T cells remain in a functionally inac-

Table 1. Cytokines involved in the pathogenesis of RA, potential therapeutic targets and targeting DMARD

Cytokine	Source cells	Cytokine function	Targeting bDMARD
Interleukin-1 (IL-1)	Monocytes, B cells, fibroblasts and chondrocytes	Promotes fibroblast cytokine release, and release of prostaglandins, matrix metalloproteinases and reactive species. Activates endothelial cell adhesion molecules, and reduces GAG synthesis. Results in increased cartilage breakdown, reduced cartilage synthesis, activation of osteoclasts with bone and cartilage damage	Anakinra
Tumor necrosis factor (TNF)	Monocytes, T and B cells, NK cells, mast cells, synoviocytes and osteoblasts	Activation of monocytes and PMN, prostaglandin release, apoptosis of PMN and T cells, increased expression of leukocyte and endothelial adhesion molecules, increased matrix metalloproteinase production, and increased free fatty acid release from adipocytes	Infliximab Adalimumab Golimumab Certolizumab Etanercept
Interleukin-6 (IL-6)	Monocytes	B cell maturation (and hence antibody production), stimulation of hepatocyte CRP production, hematopoiesis and thrombopoiesis	Tocilizumab
Interleukin-17 (IL-17)	Lymphocytes and synovial fibroblasts	Osteoclastogenesis, matrix metalloproteinase release, and inhibition of chondrocyte glycosaminoglyan synthesis, angiogenesis	LY2439821
Interleukins-12 and 23 (IL-12/IL-23)	Macrophages and dendritic cells	IL-12 is a potent inducer of IFN-γ, and promotes differentiation of T cells to Th1 cells. IL-23 promotes the production of the pro-inflammatory cytokines IL-17 and IL-22 from Th17 cells in turn promoting increased levels of TNF and IL-1β	Ustekinumab

tivated, hyporesponsive state. A naturally occurring attenuator of T cell activation is CYTOTOXIC T lymphocyte antigen 4 (CTLA4), which is homologous to CD28 and binds to CD80/86 with tenfold greater AFFINITY. Current concepts suggest that the inhibition of COSTIMULATION of T cells is the key mode of action of CTLA4. As well as impacting on antigen presentation, CTLA4 appears to inhibit the RANKL system and TNF-mediated differentiation of MONOCYTES to osteoclasts and may explain part of the anti-erosive effect of abatacept in RA [5]. Abatacept is a fusion molecule of the extracellular domain of CTLA4 and IgG1 Fc domain, which binds to CD80/86 preventing costimulatory binding on naïve T cells, resulting in anergy. Because abatacept predominately targets naïve T cells, other pathways are less likely to be affected, preserving INNATE IMMUNITY and reactivation of memory T cells [6].

The RA paradigm has broadened from a predominantly T helper 1 (Th1) cell-mediated disorder to a model in which Th17 (IL-17 producing) cells are also crucial. Th17 cells derive from naïve $CD4^+$ cells under the influence of IL-23, producing IL-17, IL-22, IFN-γ, and TNF (Tab. 1). IL-17 drives neutrophil differentiation and maturation; activation of NEUTROPHILS, MONOCYTES and synovial fibroblasts; and increases PROSTAGLANDIN and matrix metalloproteinase synthesis. The cumulative effects of IL-17 suggest a potent role for this cytokine in joint damage. Th17 cells are induced to produce IL-17 by IL-6, IL-23 and TGF-β. The latter, in combination with IL-6, promotes differentiation into Th17 cells, but alone favors the induction

of T REGULATORY CELLS. IL-6 and IL-23 can induce Th17 cells to produce IL-22, with the latter shown to promote INFLAMMATION in the skin, but its production may be modulated in the synovium by the opposing effect of TGF-β. IL-6 additionally is important in B cell maturation and therefore production of AUTOANTIBODIES (rheumatoid factor, anti-CP ANTIBODIES), the hepatic production of C reactive protein (CRP) and hepcidin (responsible for the anemia of RA), and affects the HPA axis to cause fatigue. IL-6 is therapeutically targeted by the anti-IL-6R ANTIBODY tocilizumab. The growing importance of the IL-17/IL-12/IL-23 axis in RA is demonstrated by the development and process to trials of the specific anti-IL-17 monoclonal ANTIBODY LY2439821, and ustekinumab, which targets both IL-12 and IL-23, for this indication (Tab. 1).

CYTOKINES, CHEMOKINES, ANTIBODIES/ANTIGENS, and cell-cell interaction *via* signaling pathways leads to altered GENE EXPRESSION, stabilization of mRNA, or the cell being permitted to change its activation status for a particular response. These coordinated intracellular signal transduction cascades utilize the NF-κB pathway, MITOGEN-activated protein (MAP) kinases, Janus tyrosine kinases (JAK)/signal transducers and activators of transcription (STAT), and spleen tyrosine kinase (Syk). Because of the cross-signaling and AMPLIFICATION loops involved, targeting a component of one pathway may also lead to inhibition of another pathway. These enzymes are amenable to inhibition by small molecules, which can be produced using lower cost techniques compared to bDMARD [7].

The majority of RA patients will be treated with a succession of DMARD and bDMARD alone, or in combinations that are immunosuppressant. Their profound effects on the IMMUNE SYSTEM result in an increased risk of acquired and reactivated infection. Pragmatically, all RA patients should be screened, before treatment, for tuberculosis, hepatitis B and C virus, with updated screening if an exposure risk has occurred.

DMARD and bDMARD

The most commonly used traditional DMARD is METHOTREXATE and, consequently, particular attention has been paid to this agent in this chapter. Other widely used DMARD are LEFLUNOMIDE, SULFASALAZINE and the antimalarial, hydroxychloroquine. The gold complexes have less current use, although they are clearly active. Azathioprine and CICLOSPORIN are minor drugs for the treatment for RA, although they are used sometimes in combination with other DMARD. The rapidly expanding array of bDMARD is being utilized much earlier in the treatment of RA often in conjunction with METHOTREXATE, effectively replacing other DMARD.

METHOTREXATE monotherapy has become the standard first-line treatment for RA. Subsequent treatment is more variable and depends upon a variety of factors including concomitant diseases and the severity of the RA. If the response to METHOTREXATE is insufficient, then another DMARD is often added quickly and, if the EFFICACY is still inadequate, a bDMARD is frequently used with METHOTREXATE. Combinations, particularly with METHOTREXATE, are recommended on the basis of systematic randomized controlled trials (RCTs) showing that combination treatment is more effective in both early RA and late RA (see *Combination DMARD therapies* below). The British guidelines [8] exclude a bDMARD for first-line therapy as not cost effective. In contrast, the American College of Rheumatology (ACR) guidelines recommend METHOTREXATE combined with a bDMARD for early and active RA, possibly reflecting difference in health funding [9].

Conventional DMARD therapy results in up to 15% of RA patients achieving a sustained DMARD-free remission, which is predicted by acute onset of symptoms, shorter duration before treatment, non-smoker status, IgM rheumatoid factor negativity, absence of HLA shared epitope alleles, and minimal radiographic damage at baseline [10].

Clinical measures used in the evaluation of anti-rheumatic drugs

ACR20/50/70: Clinical trials in RA commonly use the ACR percentage response developed by the American College of Rheumatology. The ACR20 score is the percentage of the cohort of interest who achieves a 20% improvement in tender and swollen joint

count plus 20% improvement in at least three of five of the following criteria: patient pain assessment, physician global assessment, patient global assessment, patient self-assessed disability and acute-phase reactant concentrations in plasma. While a reliable discriminator between active and placebo in clinical trials, the ACR20 represents only a small clinical improvement, with ACR50 and ACR70 scores being more clinically meaningful. As a percentage change, it requires a comparison in clinical status before and during treatment.

Disease activity score (DAS) was developed by the European League Against Rheumatism. Like the ACR scores, the DAS is a composite score calculated from a count of active and swollen joints, inflammatory marker levels (CRP, erythrocyte sedimentation rate), and patients general health rating from a visual analogue scale. This has the advantage of providing individualized values that give a result at any point in time. A disease activity score derived from assessing 28 joints (DAS28) above 5.1 corresponds to high disease activity, a score ≤3.2 representing low disease activity, while a score <2.6 is a validated measure of remission.

Earlier therapeutic intervention has been combined with strategies to achieve a pre-determined TARGET of minimum disease activity utilizing these composite outcome measures, or a simpler pragmatic approach of aiming at NORMALIZATION of inflammatory markers and lowest possible count of swollen and tender joints.

Radiographic progression commonly utilizes the modified Sharp score, which scores both joint space narrowing and erosions in the hands and feet. Results are presented as total scores, joint space scores and erosion scores.

Difficulties in evaluating the therapeutic efficacy of anti-rheumatic drugs

The RCT is the standard method of evaluating the EFFICACY of drugs and the results of such trials on antirheumatic drugs are quoted widely in this chapter. As examples, comparisons of treatments including active drug *versus* placebo or between two or more active drugs are discussed. However, the results of RCTs often do not indicate the overall benefit of antirheumatic drugs in clinical practice. Many patients treated with anti-rheumatic drugs in normal clinical practice have less active disease than patients in RCTs [11]. Other problems of RCTs in RA include the often constant dosage regimens, the lack of published data on individual patients, the use of single drugs, and, because of the limited number of patients in a clinical trial, the inability to detect rare adverse reactions. RCTs are, of course, still of great value but they do have limitations.

bDMARD

As outlined above (see *Pathophysiology of RA*), several CYTOKINES have major pro-inflammatory actions and are also involved with the systemic effects of RA. Thus, the neutralization of these agents leads to profound inhibition of INFLAMMATION in joints. Several anti-CYTOKINES are now available or in clinical trial for the treatment of RA (Tab. 2). These anti-CYTOKINES have revolutionized the treatment of RA and other inflammatory diseases. Indeed, their success is leading to an unprecedented phase of investigation on new drugs for the treatment of RA. As proteins, bDMARD require parenteral administration either by subcutaneous injection or intravenous infusion.

The available anti-cytokine biological agents are effective in the treatment of RA either when used alone or in combination with other DMARD, particularly METHOTREXATE. Overall, about two thirds of patients respond with a clinically significant degree of disease control. The bDMARD, ETANERCEPT, ADALIMUMAB, INFLIXIMAB, abatacept, and rituximab generally have similar clinical EFFICACY; ANAKINRA is less effective [12]. Compared to traditional DMARD, bDMARD generally show a more rapid response with slowing of the radiographic progression of RA. Combinations of TNF antagonists with low-dose METHOTREXATE have generally been more effective than either drug alone in the treatment of RA (see *Combination DMARD therapies* below).

Table 2. Monoclonal antibodies used as bDMARD. Nomenclature and structures

Substem in name	Example	Structure
-xi-	Infliximab	Mouse-human chimeric antibody The variable domains of the heavy (V_H) and light (V_L) chains of a mouse antibody to human TNF are cloned and fused to the constant domains of a human antibody, thus generating a chimeric antibody. Thus, the constant regions of the mouse antibody are removed and replaced by the constant human regions. This reduces the amount of mouse antibody sequence by approximately two thirds, but the remaining framework and complementarity determining regions (CDRs) still originate from the mouse
-zu-	Certolizumab	Humanized The human CDR sequences of an irrelevant, fully human antibody are mutated to those of the sequences of the CDR regions of a mouse antibody to human TNF. The only remaining sequences of mouse origin are the CDRs, i.e., the peptide loops located in the V domain and supported by the framework scaffold, that are responsible for contacting and binding antigen. All the structure is human apart from mouse CDRs
-u-	Adalimumab Golimumab	Fully human antibody Adalimumab – isolated through humanizing an existing anti-human TNF mouse antibody using technique termed guided selection and phage display technology. The V_H and V_L genes of a mouse antibody to human TNF are separated and sequentially paired with separate human genes for V_H and V_L domains from human antibody gene libraries, i.e., the V_H of the mouse antibody are paired with a diverse range of human V_L domains. Antibody proteins are generated and screened for binding to human TNF by phage display. Subsequent pairing of the isolated human TNF-specific V_L with a library of human V_H regions, followed by screening for optimal binding to human TNF, results in the isolation of a fully human antibody V_H and V_L domains. *In vitro* affinity maturation to increase affinity is performed by mutating CDR regions and selecting for optimal TNF binders. Selected V_H and V_L genes are subsequently fused to a human IgG1 Fc domain. The final protein is a variant of a natural fully human antibody with variation occurring in the CDR regions. Golimumab – was isolated using genetically engineered mice with human humoral immune systems, i.e., human immunoglobulin gene loci are incorporated into the genomes of the mice, so that a humoral immune response generates antibodies with human sequence. Immunization with human TNF allows the subsequent generation of mouse hybridomas secreting human TNF-specific human antibodies.

Monoclonal antibodies – bDMARD

Three different types of ANTIBODY have been used as bDMARD (Fig. 2; Tab. 2)

Pharmacokinetics

The anti-TNF ANTIBODIES have long $t_{1/2}$s, of the order of 1–2 weeks [13, 14], which allow relatively infrequent dosage (see Appendix C15). The newer agents goli-

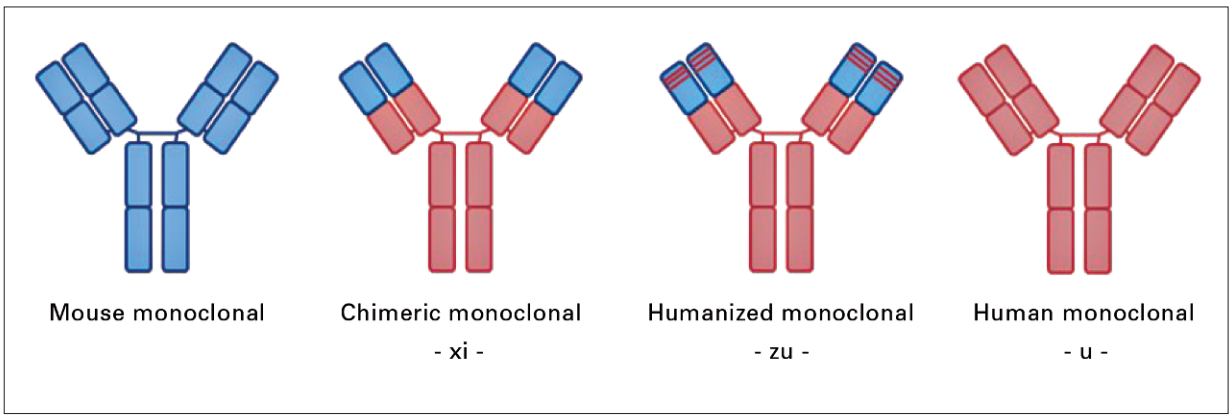

Figure 2. Diagrammatic structures of anti-bodies used as bDMARD

mumab, certolizumab, rituximab and abatacept also have long $t_{1/2}$s [15, 16], with golimumab able to be administered intravenously once every 3 months.

The half-life ($t_{1/2}$) of etanercept is somewhat shorter than the other anti-TNF products with a $t_{1/2}$ about 3–5 days [17]. Consequently, one or two dosages a week produce relatively constant plasma concentrations.

Anakinra has the greatest clearance of all the present bDMARD with an apparent $t_{1/2}$ of elimination of about 3 hours after the usual daily subcutaneous injection. Anakinra is eliminated predominately by renal excretion and its dosage must therefore be reduced in patients with severe renal impairment [18].

There is considerable inter-patient variation in the pharmacokinetics of all bDMARD. This inter-patient variation may have considerable clinical significance. The bDMARD typically have poor effect in 20–40% of patients, while some patients may respond to particular bDMARD and not others. It has been suggested that a higher than average clearance and the consequent lower plasma concentrations may, in some patients, lead to impaired anti-rheumatic response. This is a principle of the clinical pharmacology of conventional drugs but, as yet, there is only limited evidence for this relationship with bDMARD. However, infliximab has shown an improved response if trough plasma concentrations are over approximately 1 µg/mL [19–21]. Interestingly, the initial $t_{1/2}$ decreases with increasing severity of the rheumatic disease, possibly due to higher concentrations of TNF in plasma and tissues and the formation of antibody complexes with TNF.

Chemistry and targets

Tumor necrosis factor

Rationale

TNF in particular has key roles within the inflammatory network as well as heading an inflammatory cascade (Tab. 1). Anti-TNF therapy quickly suppresses leukocyte migration, deactivates endothelial cells, and promotes recovery of regulatory T cell function and phenotype.

Infliximab targets TNF and was the first chimeric (murine-human) monoclonal antibody to be approved for the treatment of autoimmune diseases. Subsequent monoclonal antibodies targeting TNF include adalimumab, golimumab, and certolizumab. Etanercept is a soluble receptor construct containing an Fc immunoglobulin backbone and a dimer of the extracellular portion of human TNF receptors (Tab. 2). It is thus a recombinant soluble TNF-α receptor fusion protein that binds both TNF and lymphotoxin, although lymphotoxin blockade may not be significant.

Clinical indications and efficacy

Therapeutic blockade of TNF yields a clinical response in ~70% of RA patients, with the 60:40:20 rule indicating that approximately 60% of patients achieve at least an ACR20 response, 40% at least an ACR50 or response and 20% an ACR70 response. Clinical response and remission are decreased in older patients, as well as patients with low functional status, and concomitant prednisolone therapy [22]. Introductory data indicate that the antirheumatic response to ADALIMUMAB increases with increasing expression of the mRNA of CD11c [23]. CD11c is a gene leading to the synthesis of INTEGRINS, proteins that are involved in the ADHERENCE of NEUTROPHILS and MONOCYTES to endothelial cells. This clinical correlation, if confirmed, may indicate a further mode of action of the anti-TNF ANTIBODIES.

Because of its CHIMERIC nature, INFLIXIMAB is used in combination with METHOTREXATE, which limits the development of neutralizing anti-drug ANTIBODIES to the mouse-derived protein material. These ANTIBODIES may be responsible for the tachyphylaxis observed in some patients treated with this agent alone. Although the other anti-CYTOKINES are not required to be administered with METHOTREXATE, they often are used with METHOTREXATE because the combination is more effective than anti-cytokine treatment alone. The same consideration is applied to clinical trials where the BIOLOGICALS are tested mostly in patients already taking older DMARD, particularly METHOTREXATE.

One of the newest anti-TNF ANTIBODIES is golimumab, which can be administered monthly subcutaneously or every 3 months intravenously. Certolizumab (certolizumab pegol) is also a new TNF blocker with a different structure to the other agents. It is a HUMANIZED Fab fragment combined to polyethylene glycol. Thus, it does not have an Fc domain. As a consequence of its structure, certolizumab does not bind to transmembrane TNF. It was thought that this would prevent some of the toxic effects of the other TNF blockers, such activation of latent tuberculosis. Unfortunately, certolizumab has led to reactivation of tuberculosis. The conjugation of peptides with polyethylene glycol (PEGYLATION) is well known to slow the elimination of peptides. In the case of certolizumab, PEGYLATION extends the $t_{1/2}$ of elimination to approximately 20 days.

While anti-TNF ANTIBODIES are the first line of bDMARD therapy, there are patients who either do not respond, have adverse events, or, due to co-morbidity, are excluded from therapy. Many of these apparent failures may be due to low plasma concentrations of the anti-TNF ANTIBODIES (see *Monoclonal antibodies – bDMARD, Pharmacokinetics* above), but research is still required to determine if a better response can be obtained by increasing the dose and hence the plasma concentrations of the anti-TNF ANTIBODIES. In apparent failure to the anti-TNF ANTIBODIES, rituximab, abatacept, and tocilizumab are considered, although METHOTREXATE background therapy is recommended for both rituximab and abatacept.

In addition to RA, the anti-TNF agents all appear in the treatment of psoriasis, PSORIATIC ARTHRITIS and ankylosing spondylitis. Although ETANERCEPT has approximately equal activity to the other anti-TNF agents in the treatment of RA, it may be less efficacious in the treatment of psoriasis [24]. Except for ETANERCEPT, the anti-TNF agents are also useful for granulomatous diseases, such as Crohn's disease, Wegener's granulomatosus and sarcoidosis.

Adverse effects

An increased incidence of infections has been reported during trials and in post-marketing surveillance, presumably related to the IMMUNOSUPPRESSIVE EFFECTS of these agents, with particular emphasis on the reactivation of chronic infections such as tuberculosis. Meta-analysis showed INFLIXIMAB and ADALIMUMAB doubled the risk of serious infection with a tendency towards a dose-response association [25]. Anti-TNF agents can reactivate latent tuberculosis and screening for tuberculosis is therefore required before treatment. There have also been reported increases in intracellular or opportunistic pathogens, including *Legionella*, *Listeria*, and *Salmonella*, with caution recommended on the consumption of raw eggs, and unpasteurised milk. In general, these agents should generally be avoided in patients with active or recurrent infections. In particular, concomitant therapy with corticosteroids may increase the susceptibility to infections. Combined treatment with

ANAKINRA and a TNF inhibitor causes an increased risk of infection, as has also been recently shown with abatacept and ETANERCEPT. These combinations are contraindicated.

It has been postulated that inhibitors of TNF could reduce immune surveillance and lead to the development of tumors. A population based study linking three RA cohorts to the Swedish cancer registry found the RA cohort was only at a marginally elevated overall risk of solid cancers. Not surprisingly, given smoking is associated with RA, smoking-related cancers were increased by 20–50%, and the risk for non-melanoma skin cancer increased by 70%. The good side was a 25% decreased risk for breast and colorectal cancer, with the cancer pattern in patients treated with anti-TNF agents mirroring historic RA cohorts [26]. The same authors observed a twofold increased risk of lymphoma and leukemia, but not myeloma. Amongst RA patients treated with anti-TNF agents the risk of lymphoma was tripled, but was not higher than in other RA cohorts [27].

In children using TNF blockers, post marketing surveillance found an increased risk of malignancy, with INFLIXIMAB having a higher reporting rate for lymphoma and all malignancies and ETANERCEPT having a higher reporting rate for lymphoma only [28]. However, a clear causal link could not be established because of confounding due to the underlying illness and concomitant immunosuppressives.

Interleukin-1

Rationale

IL-1α and IL-1β are expressed in RA synovium related to MONOCYTES, B cells, fibroblasts and chondrocytes. Mice deficient in the naturally occurring RECEPTOR antagonist (IL-1ra) develop a spontaneous erosive arthritis. ANAKINRA is very similar to IL-1ra, but is not glycosylated and also contains a terminal methionine residue, which is necessary for its biological production. ANAKINRA binds to IL-1 RECEPTORS and thus prevents IL-1 interaction with its native RECEPTOR (Tab. 1). ANAKINRA is effective in disorders that are driven by IL-1 dysregulation, such as the cold autoinflammatory disorders, but it has only been of modest EFFICACY in treating RA in adults, although it may be effective in the treatment of juvenile RA.

B cells

Rationale

Despite the presence of B cells in inflamed RA synovium and the presence of rheumatoid factor being a poor prognostic factor, B cells were not specifically targeted in the treatment of RA until the last few years. Originally developed for the treatment of B cell lymphoma, the use of anti-CD20 ANTIBODY rituximab in RA did not have a strong rationale, but was noted to improve the synovitis of an RA patient with B cell lymphoma. B cells are now realized to act as efficient antigen-presenting cells, promote T cell accumulation and provide COSTIMULATORY SIGNALS and CYTOKINES to sustain T cell activation in the RA synovium. CD20 is expressed after maturation of B cells from STEM CELLS and is lost when they mature to plasma cells. The CD20 LIGAND is an attractive TARGET for IMMUNOTHERAPY on the basis that it does not internalize upon monoclonal ANTIBODY binding and because it does not shed from the cell surface. Depletion of all CD20-bearing B cells, leaving only STEM CELLS and plasma cells, reduces autoantibody production and subsequent immune-complex formation, and reduces B cell-derived CYTOKINES such as IL-6, TNF and lymphotoxin. B cell depletion also prevents the formation of ectopic germinal centers and the optimal activation of T cells.

Rituximab is a CHIMERIC monoclonal ANTIBODY made of mouse light and heavy variable domains of the anti-CD20 ANTIBODY together with the κ-light chain domain and heavy chain constant domain of human IgG1 [29]. Rituximab leads to rapid and prolonged depletion of $CD20^+$ B cells *via* ANTIBODY-dependent cell-mediated CYTOTOXICITY, complement-dependent CYTOTOXICITY, and induction of B cell APOPTOSIS [29,30].

Ocrelizumab and ofatumumab are HUMANIZED and fully human anti-CD20 MONOCLONAL ANTIBODIES, respectively, and are presently in clinical trial. Other strategies for modifying B cell activation and survival involve targeting of the B cell proliferation-inducing

LIGAND (APRIL) and B cell-activating factor (BAFF), although the latter has not been successful in RA patients to date.

Clinical indications and efficacy

Controlled trials have demonstrated the EFFICACY of rituximab in RA, with the European Medicines Agency recommending it be considered in patients with clinically active RA with an inadequate response or intolerance to at least one other DMARD, including one or more anti-TNF agents. The recommended dose is 1 g repeated 14 days later, and is different to the lymphoma schedule of 375 mg/m^2 given weekly for 4 weeks [31]. Rituximab is given in combination with weekly METHOTREXATE 10–25 mg, which improves the clinical response. This recommendation obviously limits the use of rituximab to patients who tolerate METHOTREXATE.

Peripheral B cells count can be monitored during treatment with rituximab, with only partial depletion predicting a poor clinical response. The repopulation with B cells can be detected by highly sensitive technique of fluorescence-activated cell sorting (FACS), but the dilemma is whether rituximab should be administered again on the return of B cells or active RA, or what to do if disease returns without easily measurable B cell repopulation. The repeat dosing with rituximab at approximately 6-month intervals may be an effective option, with EFFICACY being observed after subsequent rituximab courses comparable to that seen during the initial course, irrespective of prior anti-TNF exposure. Further improvement has been noted with subsequent courses even in patients who did not respond well to the first course of treatment. These patients often have higher numbers of preplasma cells before treatment and may respond to an earlier retreatment with rituximab [32].

Adverse effects

In the clinical trials of rituximab, the overall frequency of adverse events and serious adverse events was similar in both rituximab- and placebo-treated groups. A slightly higher proportion of patients receiving rituximab experienced an infusion reaction (23%) compared with placebo (18%), and this was less at the second infusion. The frequency of adverse events remained stable after the first, second, third, and fourth courses. Reduction in serum IgM concentration, and to a lesser extent IgG, was observed in some patients following repeated courses of therapy; however, there was no link to an enhanced infection risk.

The risk of developing bacterial and viral infections with rituximab therapy was not significantly different to those taking other DMARD, and was predominantly recorded as respiratory tract bacterial infection. Serious infections occurred in 2.3% of rituximab-treated patients and 1.5% of controls, which is similar but less than the 3.7% seen in anti-TNF-treated RA patients. Viral load increases in patients with hepatitis C-related mixed cryoglobulinemia, reinforcing the need for screening, and dose modification and/or concomitant antiviral therapy is required.

Interleukin-6

Rationale

IL-6 is a monocyte-derived cytokine important in B cell maturation (and hence ANTIBODY production), stimulation of hepatocyte CRP production, HEMATOPOIESIS and thrombopoiesis. IL-6 signals primarily through a membrane-bound complex of the non-signaling IL-6 RECEPTOR (IL-6R) and two signal-transducing gp130 subunits. IL-6R is predominantly expressed on NEUTROPHILS, monocyte/MACROPHAGES, hepatocytes and some LYMPHOCYTES. However, soluble (s) IL-6R can be generated by proteolysis of the membrane-bound IL-6R or by alternative mRNA splicing. Released sIL-6R is able to complex with IL-6 and be transported in bodily fluids, with the IL-6:IL-6R complex able to bind and activate the ubiquitously expressed signaling gp130 subunit, which is found on a range of cells such as endothelial cells and synoviocytes. Thus, IL-6 has predictable actions *via* IL-6R-bearing cells, but has a broader potential effect on any cell expressing gp130.

IL-6 mobilizes marginated NEUTROPHILS into the circulation, with large numbers of NEUTROPHILS found

in synovial fluid. Binding of IL-6 to neutrophil membrane-bound IL-6R induces secretion of proteolytic enzymes and reactive oxygen intermediates causing cartilage degradation. During acute INFLAMMATION in RA, MONOCYTES, MACROPHAGES and endothelial cells release IL-6 accompanied by an increase in NEUTROPHILS in synovial fluid. Subsequently, IL-6 is thought to influence the shift from acute to chronic INFLAMMATION, marked by an increase in the recruitment of MONOCYTES.

IL-6 is the principal stimulator of acute-phase protein synthesis through hepatocyte stimulation, with levels correlating with CRP levels in RA patients. Hepatocyte production of hepcidin is stimulated by IL-6 preventing iron transport and the release of iron from MACROPHAGES causing anemia of chronic INFLAMMATION. Tocilizumab is a HUMANIZED monoclonal ANTIBODY that binds both soluble and membrane-expressed IL-6R, limiting IL-6 pro-inflammatory activities through inhibition of the gp130 pathway [33].

Clinical indications and efficacy

The IL-6 RECEPTOR antagonist, tocilizumab, has a licensed dose of 8 mg/kg infused over 1 hour every 4 weeks. In clinical trials 2 mg/kg, 4 mg/kg, and 8 mg/kg were infused intravenously every 4 weeks, usually with background weekly METHOTREXATE. In RA patients who had failed to respond or had been intolerant to an anti-TNF agent within the previous year, dosage with tocilizumab (8 mg/kg) plus METHOTREXATE was superior to METHOTREXATE alone [34].

Adverse effects

Events particularly related to tocilizumab are NEUTROPENIA, increase in hepatic enzymes, cholesterol elevation, and caution in patients with a previous history of intestinal ulceration or diverticulitis.

Reduction in neutrophil count occurs more frequently in those with high baseline values and very frequently in those with normal baseline values. Few counts go below 1000 cells/mm^3, and there was no association between low neutrophil counts and infection-related serious adverse reactions. In trials both tocilizumab interruption and DMARD dose modification were used, with a recommendation not to treat with neutrophil counts $<0.5\times10^9$/L, or platelet counts $<50\times10^9$/L. Elevation of transaminases (AST and ALT) up to three times the upper limit of normal occurs three times more often than that seen with DMARD only, with highest mean increases 2 weeks after each infusion. More than threefold elevation of enzymes is much less prevalent (2–4%), but remains three to five times higher than with DMARD alone. Total bilirubin increase within three times the upper limit of normal is also seen. It is recommended to be cautious in the use of tocilizumab in patients with active liver disease or elevated hepatic enzymes (50% above the upper limit of normal) and not to treat patients with ALT or AST more than five times the upper normal levels). Transaminases should be monitored every 1–2 months during the first 6 months of therapy and every 3 months thereafter.

Upper gastrointestinal events suggestive of INFLAMMATION, gastritis or ulcer are also more common. Increases in fasting plasma lipids occurs early after treatment and remain elevated during therapy with approximate mean changes: total cholesterol 0.8 mmol/L, HDL cholesterol 0.1 mmol/L, LDL cholesterol 0.5 mmol/L. Approximately 24% of patients receiving tocilizumab experienced sustained elevations in total cholesterol to at least 6.2 mmol/L. Lipid parameters should be measured 4–8 weeks after commencing therapy and managed according to local guidelines and taking into account the individual risk factors.

The rates of tuberculosis do not seem increased, but all RA patients should be screened for latent disease.

Second costimulatory signals

Rationale

As outlined above (see *Cytokines in RA*, Tab. 1), T cells are implicated in the pathogenesis of RA. Abatacept (CTLA4-1g) is a fusion protein produced from CYTOTOXIC T LYMPHOCYTE-associated antigen 4

(CTLA4) with the Fc portion of IgG1 [35]. Abatacept binds to CD80/CD86 on the surfaces of B cells and MONOCYTES leading to decreased activation of T LYMPHOCYTES and preventing the release of inflammatory mediators [35].

Clinical indications and efficacy

Abatacept is administered intravenously at 0, 2, and 4 weeks and then every 4 weeks (<60 kg: 500 mg dose, 60–100 kg: 750 mg dose, and >100 kg: 1000 mg dose). Background therapy with METHOTREXATE is recommended.

Monotherapy with abatacept is effective in refractory RA previously treated with one or more DMARD [36]. After 2 years of treatment, 50% of patients had no progression of structural damage, with a suggestion that the disease-modifying effect may increase over time [37].

Adverse effects

Abatacept is well tolerated but should not be administered in combination with ETANERCEPT due to increased serious adverse events, including serious infections, without any significant additional EFFICACY [38].

Interleukins-12 and -23

Rationale

The involvement of IL-12 and IL-23 is described above (see *Cytokines in RA*, Tab. 1). Ustekinumab is the fully human IgG1κ monoclonal ANTIBODY, anti-IL-12p40, against the p40 subunit common to both IL-12 and IL-23, which prevents interaction with the IL-12 Rβ1 RECEPTOR. On the basis of IL-12 and IL-23 expression in lesions, trials have been initiated in psoriasis and MULTIPLE SCLEROSIS. Significant EFFICACY has been shown in chronic plaque psoriasis, with promising results in early PSORIATIC ARTHRITIS studies, but of no value in MULTIPLE SCLEROSIS. There have been insufficient studies to date to make any definite conclusion about its activity in RA.

General adverse effects of bDMARD

Monitoring

Detection of potential adverse effects is an integral part of each consultation, reviewing recent and current events, and anticipating potential effects related to new prescribing, overseas travel, or surgery. Any suspected sepsis or exposure risks should be thoroughly characterized, as bDMARD may significantly ameliorate both clinical and laboratory indicators of severity. RA patients have increased risks of cardiovascular events, smoking-related malignancy, and skin cancers and lymphomas. Monitoring should include 6–12 monthly skin and lymph node examination; 6–12 monthly blood pressure, glucose, and fasting lipid profiles, and monthly renal and liver function as indicated by concomitant medication. Patients having received B cell-depleting therapy may require measurement of B cell repopulation and immunoglobulin levels.

Progressive multifocal leukoencephalopathy

Progressive multifocal leukoencephalopathy (PML) is a very rare, but usually fatal, CNS infection caused by the JC polyoma virus, which leads to demyelination. JC polyoma virus is a common and usually clinically silent human infection, which reactivates with IMMUNOSUPPRESSION and leads to mental, motor and visual decline with a final rapid decline to coma and death. T cell lymphopenia, HIV INFECTION, and immunosuppressive therapy are primary risk factors, with cases reported in SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) and RA patients receiving conventional immunosuppressive therapy. PML has been reported in RA patients treated with INFLIXIMAB, ETANERCEPT, and tocilizumab [39]. In some cases reactivation of virus has been proven, but it has been suggested that the treatment itself may be associated with non-infectious demyelination, with a recommendation that the polymerase chain reaction test (PCR) be performed on the cerebrospinal fluid of all RA patients treated with newer

immunosuppressive agents who present with a demyelination syndrome. It is also recommended that bDMARD should not be used in patients with known pre-existing demyelinating conditions such as MULTIPLE SCLEROSIS.

In theory, B cell depletion should have no effect on activation of this virus, but sufficient confirmed or suspected cases have been reported, primarily in oncology patients, for the manufacturer in Europe to recommend all rituximab-treated patients to carry a "Patient Alert Card". The cumulative reporting rate of 2.2 cases per 100 000 RA patients treated with rituximab is approximately double the expected cumulative incidence rate in RA of 1 per 100 000 [40], and it has been proposed that post-marketing surveillance of patients taking rituximab or other bDMARD is essential. Patients should be made aware of the risk of this infection and informed consent is strongly recommended before treatment with this bDMARD is commenced [30].

Induced autoimmunity

CHIMERIC ANTIBODIES are recognized as foreign by the human IMMUNE SYSTEM and may provoke an allergic reaction, and the development of human anti-CHIMERIC ANTIBODIES (HACA) may decrease the activity or increase the CLEARANCE of the ANTIBODY. Notwithstanding the increased "humanness" of human and HUMANIZED ANTIBODIES, they remain immunogenic, and a small proportion of users develop human anti-human ANTIBODIES (HAHA). ANTIBODY production against bDMARDs is reduced by concomitant use of METHOTREXATE, presumably on the basis of its immunosuppressive activity [41].

DMARD and bDMARD have been associated with the development of alternative autoimmune disorders, such as lupus-syndromes reported with LEFLUNOMIDE and TNF blockers, and psoriasis in patients treated with rituximab. The use of anti-TNF agents has been associated with formation of anti-nuclear ANTIBODIES and anti-double-stranded DNA ANTIBODIES. However, lupus reactions, unless there is existing end-organ damage, are very rare and there is presently no case for testing for these ANTIBODIES [42].

Infusion reactions

Treatment for anaphylactic reactions should be available for all infusion therapies, and closer monitoring is required during the initial infusions. Immunogenicity induced by biological therapies decreases with increasing humanness of the product, but remains. Immunogenicity and ANTIBODY formation is associated with acute or delayed allergic reactions. Acute infusion reactions occurred in 23% of patients within the ACCENT study (placebo around 10%), with 4% classified as severe, and 2.3% experiencing delayed HYPERSENSITIVITY [42]. Use of concomitant immunosuppressives reduces the rate of infusion reactions, and it is important not to exceed the recommended rate of infusion. If a reaction has occurred, pretreatment with ANTIHISTAMINE and corticosteroid should be considered before subsequent doses.

B cell lysis with rituximab can be associated with fever, chills, nausea and, in severe cases, hypotension and chest pain. To reduce both the incidence and severity of reactions, pre-medication with an antipyretic, ANTIHISTAMINE, and 100 mg intravenous methylprednisolone is recommended. Infusion reactions from abatacept and tocilizumab occur only in a small proportion of persons, and are usually mild requiring no pre-medication.

DMARD

Methotrexate

METHOTREXATE is a folate analogue originally developed in the 1940s as a CYTOTOXIC DRUG for the treatment of various tumors. An older folate analogue, aminopterin, was shown to be useful in treatment of RA in 1951 [43], and its replacement with METHOTREXATE led ultimately to the testing of METHOTREXATE in the treatment of RA. METHOTREXATE is, like its close analogue folic acid (Fig. 3), a hydrophilic ionized compound at physiological pH, indicating that it should not diffuse passively through cell membranes. Several transporters have been identified recently (see below).

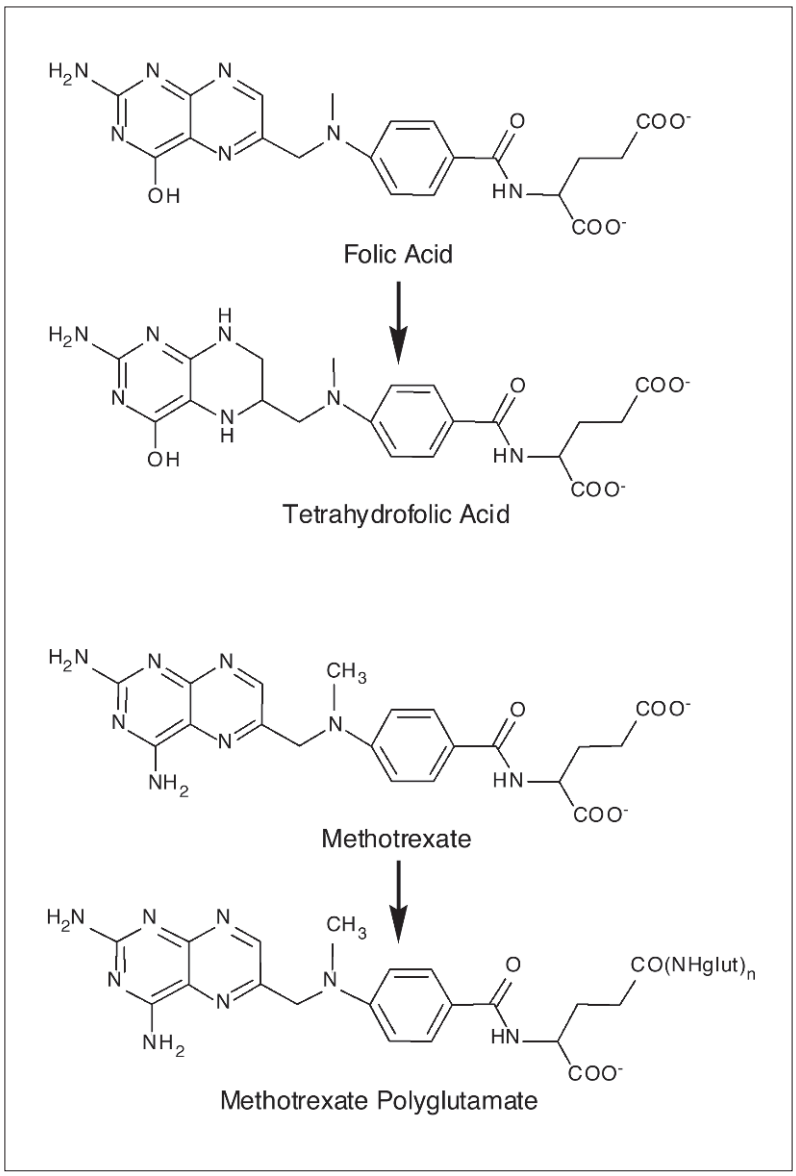

Figure 3. Comparative structures of folic acid and methotrexate
Folic acid is only active as a cofactor for one-carbon transfers after reduction to tetrahydrofolic acid. Both folic acid and methotrexate form polyglutamates in cells, as is shown for methotrexate.

Pharmacokinetics and metabolism

Methotrexate is usually administered orally but may also be administered by subcutaneous or intramuscular injection if excessive nausea occurs when the drug is taken orally and the nausea is not controlled by folic acid treatment.

Urinary recovery of methotrexate indicates that the bioavailability is about 80%. Following oral, subcutaneous or intramuscular dosage, the initial $t_{1/2}$ of methotrexate is about 7 hours. This is followed by a very slow phase with a $t_{1/2}$ about 5 days [44]. This slow elimination phase, possibly caused by the slow intracellular accumulation and loss of the active

polyglutamate metabolites (see below), probably serves to prolong the effects of METHOTREXATE and to allow for its once-a-week dosage in RA.

METHOTREXATE is primarily eliminated *via* the kidneys. Therefore, a lower dose of METHOTREXATE should be used in patients with chronic renal impairment and temporary cessation of METHOTREXATE treatment may be required at times of volume depletion (such as perioperatively). Dosage should also be decreased in older age because of decreasing renal function. Co-prescription of agents known to impair renal function, such as aminoglycosides and CICLOSPORIN should be undertaken with caution. It has also been reported that prolonged use of METHOTREXATE itself may reduce renal function and hence its own CLEARANCE [45], a possible mechanism being increased plasma adenosine concentrations as a consequence of METHOTREXATE-activating A_1 RECEPTORS in the renal parenchyma, thereby diminishing renal blood flow and salt and water excretion [46].

Many patients taking low-dose METHOTREXATE are also treated with NSAID to suppress the symptoms of INFLAMMATION, although toxicity from METHOTREXATE in occasional patients has been attributed to this combination of drugs. Although renal blood flow and renal function can be decreased by NSAIDs, prospective studies do not indicate any NSAID-induced decrease in the renal CLEARANCE of METHOTREXATE, except during treatment with high doses of aspirin [47]. Probenecid decreases the renal excretion of METHOTREXATE and should be avoided [48]. Additionally, BONE MARROW suppression has occasionally been seen with the combination of cotrimoxazole and METHOTREXATE, probably because cotrimoxazole has weak anti-folate activity in humans.

METHOTREXATE contains a glutamate moiety and, after entering the cell, up to six glutamates are added by the action of folyl-polyglutamyl synthase (Fig. 3). This polyglutamation maintains a low intracellular concentration of METHOTREXATE as the polyglutamates cannot be transported extracellularly, unless hydrolyzed to the monoglutamate (i.e., METHOTREXATE), by polyglutamate hydrolase. Thus, the polyglutamation of METHOTREXATE effectively increases its intracellular life and enhances its enzyme inhibitory potency because the polyglutamates are active inhibitors of dihydrofolate reductase.

Given the variable antirheumatic activity and adverse reactions of METHOTREXATE, correlations have also been sought between the clinical effects and the concentrations of METHOTREXATE or its glutamates in plasma or red blood cells. However, no consistent correlates have been found [49, 50]. It is possible that monitoring of plasma or blood concentrations early in treatment with METHOTREXATE may indicate the probability of success or failure to treatment with this drug, i.e., before the dosage of METHOTREXATE is increased in non-responding patients in attempts to improve the clinical response.

Genetic variants of enzymes or transporters have been sought as predictors of the response to METHOTREXATE but no single variant has been associated with response or failure to treatment [51, 52]. Inter-patient differences in the expression of enzymes or transporters (i.e., the level of enzyme or transporter protein) could be major causes of clinical variation in the response to METHOTREXATE, but data are lacking.

Mechanism of action

In the treatment of tumors, the major action of METHOTREXATE is inhibition of dihydrofolate reductase, the result being the blockade of the intracellular production of reduced tetrahydrofolate, which is important in the transfer of one carbon units. These are necessary for the synthesis of some amino acids and nucleic acid bases. An action on dihydrofolate reductase is also indicated in RA because the trough concentration of unbound METHOTREXATE exceeds the approximate dissociation constant of METHOTREXATE from dihydrofolate reductase [53].

Based on its actions on tumors, the mechanism of action of METHOTREXATE in RA was postulated initially as the inhibition of the proliferation of activated LYMPHOCYTES. There is, however, no convincing evidence that lymphocyte proliferation is inhibited in RA patients. More recently, it been suggested that low-dose METHOTREXATE may inhibit the recruitment of immature and inflammatory MONOCYTES into inflammatory sites and reduce their survival in the inflamed synovium, but with little or no effect on tissue-infiltrating MONOCYTES and resident MACROPHAGES [54].

Current hypotheses suggest that the beneficial effects of low-dose METHOTREXATE are secondary to an

anti-inflammatory action rather than its anti-proliferative action. In general, low-dose METHOTREXATE alters the cytokine balance by inhibiting the production of pro-INFLAMMATORY CYTOKINES (TNF, IL-6) and enhancing anti-INFLAMMATORY CYTOKINES (IL-1ra).

The major anti-inflammatory effect of low-dose METHOTREXATE may be inhibition of the enzyme AICAR transformylase, the result being the intracellular accumulation of adenosine monophosphate (AMP) and its conversion to adenosine in the extracellular space (Fig. 4). It is suggested that the higher levels of extracellular adenosine then binds to the transmembrane G protein-coupled adenosine cell surface RECEPTORS (A_1,$A_{2\alpha}$,$A_{2\beta}$ A_3) [46,55]. According to this hypothesis, METHOTREXATE predominantly acts *via* ligation of the $A_{2\alpha}$ RECEPTORS that are present on NEUTROPHILS, macrophage/MONOCYTES, LYMPHOCYTES and BASOPHILS. Binding increases intracellular cAMP, leading to IMMUNOSUPPRESSION by inhibition of PHAGOCYTOSIS; inhibition of secretion of TNF, IFN-γ, IL-2, IL-6, and IL-8, and HLA expression; and increased secretion of IL-10, an anti-inflammatory cytokine. Binding of adenosine to A_3 RECEPTORS on macrophage/MONOCYTES leads to inhibition of secretion of TNF, IL-12, IFN-γ, and IL-1ra [46, 55]. Results on A_{2A} and A_3 knockout mice are consistent with adenosine mediating the anti-inflammatory effects of METHOTREXATE because METHOTREXATE does not have anti-inflammatory activity in mice lacking either RECEPTOR [56]. Further, METHOTREXATE increases adenosine concentrations in air pouch exudates, a model of INFLAMMATION. By contrast, METHOTREXATE does not

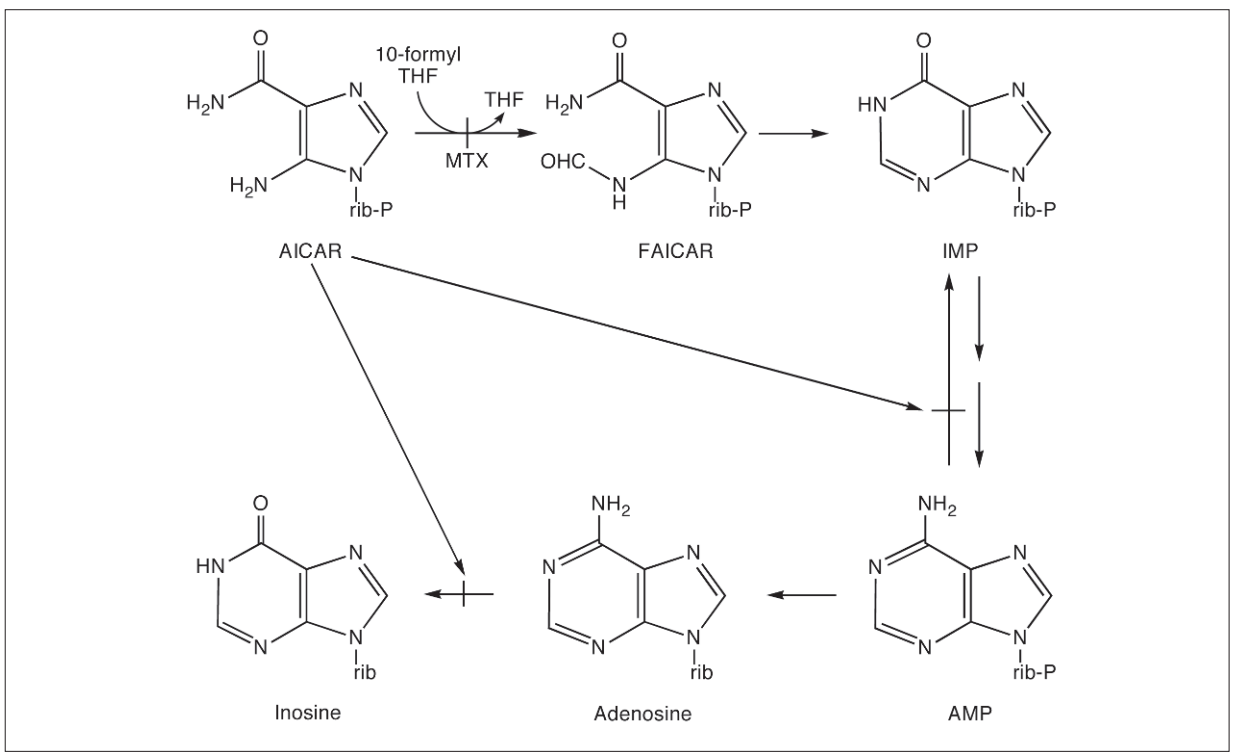

FIGURE 4. PROPOSED MODE OF ACTION OF METHOTREXATE

Methotrexate (MTX) inhibits the 5-amino-4-carboxamide ribonucleotide (AICAR) transformylase, which catalyzes the conversion of AICAR to N*-formylAICAR (FAICAR). This leads to the accumulation of AICAR, which decreases the activity of intracellular enzymes that deaminate adenosine monophosphate (AMP) and adenosine. The consequent accumulation of adenosine interacts with adenosine receptors causing suppression of inflammation.*

increase the blood concentration of adenosine in patients, although changes at peripheral sites cannot be excluded [57].

Clinical indications and EFFICACY

METHOTREXATE is an established DMARD for RA, psoriatic arthropathy and other inflammatory joint diseases. METHOTREXATE is used in the treatment of RA at lower doses than when used as a CYTOTOXIC DRUG. A recent review of dosing strategies found a starting dose of 15 mg/week orally, escalating at 5 mg/month to 25–30 mg/week or the highest tolerable dose, yields the best results [58]. Overall, the aim is to increase the dosage up to a level that produces satisfactory suppression of the activity of the disease with limited adverse effects

An outline of recent evaluations of METHOTREXATE and other DMARD (Tab. 3) indicates the required quality of modern clinical trials in RA. Measures of symptoms, clinical signs and X-ray examination are all required. In terms of the results, these trials indicate little difference between METHOTREXATE and other DMARD [59–63]. The initial response is greater for both LEFLUNOMIDE and the bDMARD, ETANERCEPT, than during treatment with METHOTREXATE, but it is unclear whether this translates into a longer term benefit. An important indication of the long-term therapeutic benefit of DMARD is their effect on the degree of damage to joints. Present evidence is that METHOTREXATE retards, but does not entirely block, joint damage in many patients (Tab. 3).

The utility of METHOTREXATE is seen from the high maintenance on treatment, with about 60–70% of patients still taking METHOTREXATE 6 years after initiation of the treatment [64, 65]. This retention rate is generally greater than seen with the older DMARD [65].

The present view on METHOTREXATE is that it should be considered for all patients at the time of diagnosis of RA. Individual factors such as pregnancy, and alcohol intake (see *Adverse effects* and *Monitoring* below) may impact on that decision, but METHOTREXATE needs to be considered. METHOTREXATE is frequently used with other DMARD depending upon the progress of the treatment of RA (see *Combination DMARD therapies* below).

Adverse effects

Low-dose METHOTREXATE produces a large number of adverse reactions (see Appendix). Early initiation of combination therapies that utilize higher METHOTREXATE doses with folic acid supplementation provide a minor caution on interpreting toxicity data from a systematic review of adult RA patients treated with METHOTREXATE monotherapy for more than 2 years, which reported favorable long-term safety. Of patients treated with METHOTREXATE, 10–35% cease therapy due to toxicity, which is less than for SULFASALAZINE and gold complexes but higher than for hydroxychloroquine. There does not appear to be an increased risk of serious infections, including herpes zoster, and the drug could provide a reduced cardiovascular mortality. The prevalence of raised liver enzymes to above twice the upper limit of normal is around 13–15%, with the data on liver fibrosis/cirrhosis conflicting. There is no strong evidence of increased lymphoma or malignancy risk but the data are suboptimal to draw strong conclusions.

Oral ulceration, nausea and fatigue occur very frequently and are probably related to intracellular depletion of folates, resulting in increased adenosine and homocysteine; hence, the usefulness of supplementation with oral folic acid. Various doses of folic acid have been recommended but present advice is that a single dose of 5 mg folic acid should be administered to all patients on the morning following the dose of METHOTREXATE [66]. Supplementation at this level does not reduce the antirheumatic EFFICACY of low-dose METHOTREXATE. Folinic acid contains the fully reduced form of folic acid and is used to treat METHOTREXATE-induced hematotoxicity and overdose with the drug.

An unexpected side effect of METHOTREXATE is the accelerated formation of rheumatoid nodules, particularly around the fingers. This may be due to activation of adenosine A_1 RECEPTORS leading to the development of multinucleated giant cells and the nodules [67]. Colchicine may prevent their formation [68]. Interstitial pneumonitis is a serious side of METHOTREXATE and occurs in 2–7% of patients. It is potentially fatal. Treatment consists of cessation of METHOTREXATE, general supportive measures and high doses of corticosteroids. Although most patients

Table 3. Comparative results of clinical trials on methotrexate and other DMARD

Treatments	Duration (months)	Methotrexate dose (mg/week)	Folate supplement	ACR 20% 50% 70%	Contrasts (ACR and radiographic progression)	Health assessment questionnaire (HAQ)[1]	Ref.
Methotrexate vs leflunomide vs placebo	12	7.5–15	Yes	46% 23% 9%	Methotrexate = leflunomide > placebo by ACR and X-ray	–0.26 methotrexate 0 placebo	[59]
Continuation of trial above	24	7.5–15	Yes	48% 28% 12%	Methotrexate = leflunomide by ACR and X-ray	–0.6 leflunomide greater improvement than with methotrexate –0.37	[60]
Methotrexate vs leflunomide	12	10–15	10%[2]	65% 44% 10%	Methotrexate > leflunomide by ACR but not by X ray	–0.7 all groups	[61]
Methotrexate vs sulfasalazine vs combination	12	7.5–15	No	59%	No differences		[62]
Methotrexate vs etanercept	12	19 (mean)	Yes	65% 42% 22%	Etanercept > methotrexate but only in first 6 months		[63]

[1]A decrease in HAQ of 0.22 is considered to be the minimum clinically meaningful decrease.
[2]Folate usually started after an adverse reaction.

with METHOTREXATE-induced lung disease have a complete recovery, some have permanent lung damage. The strongest predictors for lung injury are age above 60 years, diabetes mellitus, rheumatoid pulmonary involvement, previous use of DMARD and hypoalbuminemia [69]. METHOTREXATE should not be re-introduced after recovery from pneumonitis. Although pneumonitis is clearly an adverse effect of METHOTREXATE, many reported cases of pneumonitis were the result of pulmonary infections that were not differentiated from METHOTREXATE-induced pneumonitis.

As is the case during treatment with most DMARD, pregnancy should be avoided during treatment with low-dose METHOTREXATE because of the high risk of teratogenic effects. Consequently, treatment with METHOTREXATE, as well as with other DMARD, should be stopped before conception and not restarted until after delivery. Fortunately, the disease activity generally decreases during pregnancy but exacerbations can be treated with low-dose corticosteroids.

Monitoring

METHOTREXATE should be avoided in patients with significant pre-existing liver or lung disease. Therefore, at the start of therapy, a complete blood count, a chest radiograph and liver function tests including

measurements of aspartate aminotransferase, alanine aminotransferase, albumin and alkaline phosphatase should be undertaken [9]. Tests for hepatitis B and C should be conducted in patients who are at risk of these diseases. Serum creatinine should be assayed as a measure of renal function. The complete blood count and tests of liver and renal function should be monitored every month for 6 months and subsequently every 1–2 months. Treatment with METHOTREXATE should be stopped in patients with transaminase concentrations persistently at twice the upper limit of normal or at three times the upper level of normal at any time. At this stage, measurement of plasma concentrations of METHOTREXATE does not appear to be useful in predicting significant hepatotoxicity [70]. Liver biopsy is required only for those patients who need to continue METHOTREXATE and who have sustained enzyme abnormalities.

Antimalarials (chloroquine and hydroxychloroquine)

Hydroxychloroquine and chloroquine are antimalarial drugs. Their introduction for the treatment of RA followed the chance discovery of the value of an older antimalarial, mepacrine, in the treatment of RA. Both hydroxychloroquine and chloroquine are now used in the treatment of RA, but hydroxychloroquine is the more widely used. Both are relatively small molecular weight bases (Fig. 5).

Pharmacokinetics

The BIOAVAILABILITY of hydroxychloroquine is very variable, ranging from below 20% up to 100%, but the BIOAVAILABILITY remains fairly constant within an individual [71]. The variable BIOAVAILABILITY may be responsible for much of the inter-patient variation in the response to hydroxychloroquine. An important feature of the pharmacokinetics of hydroxychloroquine and chloroquine is their extremely long terminal $t_{1/2}$ of about 40 days. This means that steady-state concentrations may not be achieved until after 3–6 months of daily dosing. The use of loading regimens may decrease the time until the onset of effect of the ANTIMALARIALS but this is not standard clinical practice.

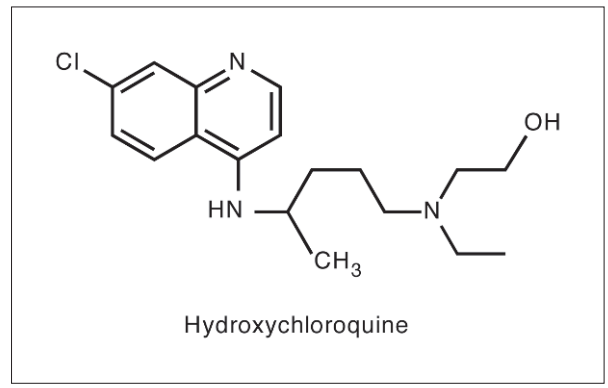

FIGURE 5. STRUCTURE OF HYDROXYCHLOROQUINE
The base structure is shown but, at physiological pH values, the molecule is very largely present as a dication with both aliphatic amino groups ionized after taking up hydrogen ions. The structure of chloroquine is the same but does not have the hydroxyl group.

Mechanism of action

The weakly basic character of the anti-malarial drugs indicates that they should accumulate in acidic organelles in cells, particularly lysosomes, where they may raise the pH. This may affect the function of the acidic organelles, particularly lysosomal enzymes. One such enzyme is acidic sphingomyelinase, which is located within the lipid membrane of lysosomes and is an important mediator in the signal transduction pathway between the TNF RECEPTOR on the cell surface and activation of transcription factor NF-κB in the nucleus [72]. Raising the pH of lysosomes inhibits acidic sphingomyelinase activity, consequently inhibiting NF-κB activity and pro-inflammatory GENE EXPRESSION. ANTIMALARIALS have also been reported to inhibit the activity of many other enzymes, including phospholipase A_2 and the production of IL-1.

Clinical indications and efficacy

ANTIMALARIALS are mild antirheumatic agents with low toxicity. This makes them attractive for use in the early stages of RA or in combination with other DMARD. However, in contrast to METHOTREXATE and

several other DMARD, the ANTIMALARIALS do not appear to retard the damage to joints produced by RA [73].

Adverse effects

Hydroxychloroquine and chloroquine have a very similar range of adverse effects, which are mostly mild and transient not requiring cessation of the drug. Hydroxychloroquine is associated with less toxicity than chloroquine. Of most concern is a rare, irreversible retinopathy, resulting in permanent visual loss. The most important risk factors are daily dosage of hydroxychloroquine and duration of therapy, with most reports of toxicity in those taking more than 6.5 mg/kg (lean weight estimation in those overweight) or taking it for over 5 years. Other risk factors are coexisting renal or liver disease, obesity, age, and pre-existing retinal disease.

Monitoring

It is recommended that there should be a baseline eye evaluation and examinations of the fundi and visual fields every 6–12 months. A relationship between the plasma concentrations of hydroxychloroquine and EFFICACY has been shown, indicating that there may be some value in measuring blood hydroxychloroquine concentrations to optimize dosing regimens [74]. However, adjusting the dosage of hydroxychloroquine after monitoring its plasma concentrations is still most uncommon.

Azathioprine

Azathioprine is a SYNTHETIC purine and anti-metabolite drug, which is commonly used as an immunosuppressant to prevent transplant rejection (see chapter C11). It has limited use in the treatment of RA.

Ciclosporin

Cyclosporin A is a fungal anti-metabolite, which is also widely used under the name CICLOSPORIN (INN) or cyclosporine (USAN) to prevent the rejection of transplanted tissues (see chapter C12). CICLOSPORIN is efficacious in the treatment of RA but has a low THERAPEUTIC INDEX and is rarely used alone in the treatment of RA. It has been most recently tested in combination with METHOTREXATE (see *Combination DMARD therapies* below).

Gold complexes

Gold complexes were introduced into therapy of RA after attempts to use them for the treatment of infectious diseases. The first large scale use was based on the mistaken belief that RA was a tubercular infection. Although the tubercular theory of RA has been disproven, the gold complexes are effective for rheumatoid and PSORIATIC ARTHRITIS. Gold does not form simple salts and only complexes of gold are formed. Complexes can be prepared with gold principally in two oxidation states, I and III, but all the clinically used complexes contain gold (I). Gold in this oxidation state forms complexes with thiols, such as cysteine residues in proteins, and cyanide as described below. Complexes containing gold (III) are powerful oxidants but may be present transiently *in vivo*.

Pharmacokinetics

Aurothiomalate is polymeric and highly water soluble. Consequently, it is poorly absorbed orally and must be given by intramuscular injection, which is administered once a week. The need for intramuscular injection makes the administration of aurothiomalate less convenient than other oral DMARD such as METHOTREXATE, SULFASALAZINE and hydroxychloroquine. Auranofin is much more lipid soluble and is partly absorbed following oral administration.

Gold complexes of albumin are the most significant complexes in plasma, and the total gold concentration is eliminated from plasma with an initial $t_{1/2}$ of about 5 days [75]. After injection, of the gold is excreted in urine, but some accumulates, particularly as aurosomes in which gold may be present for many years following the last dose of gold. These inclusions are probably formed by gold uptake into the lysosomes of synovial lining cells. A large propor-

tion of the gold appears in feces when oral gold is administered.

Aurocyanide is a metabolite of all gold (I) complexes. It is formed through the oxidation of thiocyanate (SCN^-) by myeloperoxidase, an important enzyme in the oxidative burst of NEUTROPHILS and MONOCYTES (Fig. 6). Thiocyanate is a normal body constituent ,which is oxidized, in part, to hydrogen cyanide. This then reacts with gold complexes to yield aurocyanide, which is a very stable complex of gold (I) [76].

Aurocyanide potently inhibits the oxidative burst of NEUTROPHILS and the proliferation of LYMPHOCYTES. Aurocyanide may thus mediate many of the antirheumatic and adverse effects of the gold complexes [76]. Unlike other gold complexes, aurocyanide enters cells readily. The intracellular site of action is unclear but may be pro-inflammatory transcription factors, such as AP-1 and NF-κB, which have cysteine residues within their DNA binding domains and bind gold [77].

Clinical indications and efficacy

The use of aurothiomalate has decreased considerably in recent years but is cheaper than the new bDMARD and may be preferable to DMARD and bDMARD in patients with a variety of risk factors, including chronic infections, recent malignancy, or failed treatment with other DMARD. Aurothiomalate has similar activity to that of SULFASALAZINE and METHOTREXATE [78]. An excellent response occurs in about a third of the patients treated with intramuscular gold complexes within the first year. Patients receiving aurothiomalate show less progression of erosions and a higher rate of erosion repair than patients without gold treatment. However, the long-term benefits of aurothiomalate are less impressive [78, 79].

The EFFICACY of orally administered complex, auranofin, during treatment for several months is well proven. However, the general conclusion is that auranofin is less efficacious than the injectable complex, aurothiomalate, and also less effective than METHOTREXATE, particularly during long-term treatment [78].

Adverse effects

Agranulocytosis and even aplastic anemia have been reported during gold therapy. Toxicity, however, is generally minor and often manageable if dosing is

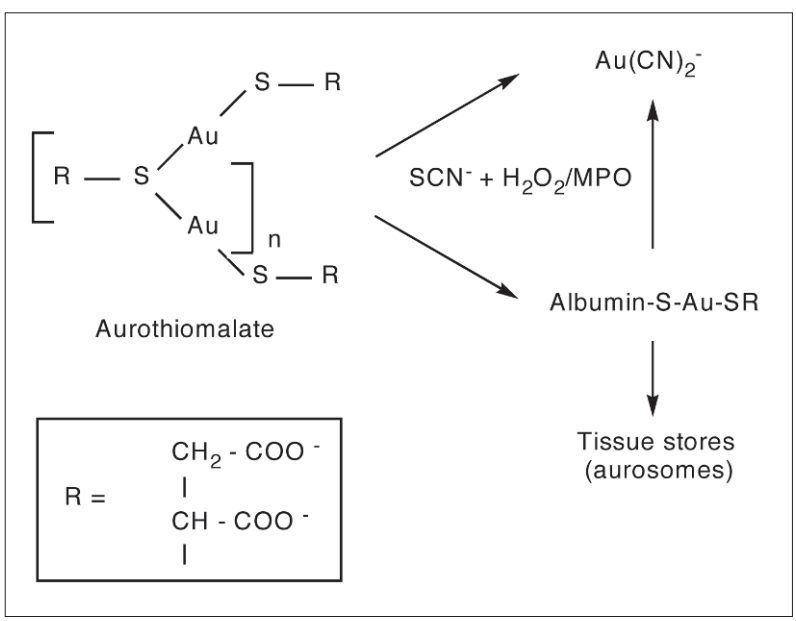

FIGURE 6. SCHEMATIC STRUCTURE AND METABOLISM OF AUROTHIOMALATE
Aurothiomalate is a polymeric compound consisting of about eight to ten units of sodium thiomalate complexed to Au(I) with the chain terminated by an additional thiomalate residue. Gold is present in plasma mainly as complexes with albumin and endogenous thiols (RS-). Aurothiomalate and albumin complexes are converted to aurocyanide [$Au(CN)_2^-$] by myeloperoxidase (MPO) during the oxidation of thiocyanate (SCN^-) by hydrogen peroxide (H_2O_2).

adjusted early upon appearance of adverse effects, although adverse effects are a common cause of withdrawals from gold therapy over 2–4 years of therapy [78]. Toxicity does not correlate with the cumulative dose.

Auranofin has a lower incidence of the side effects that are seen with the injectable gold complexes (see *Appendix*). Diarrhea is a common side effect of auranofin but not the injectable complexes. Diarrhea may decrease with continued dosage of auranofin and reduced dosage is often sufficient to stop it.

Monitoring

Before starting injectable gold therapy, a baseline evaluation should include a complete blood cell count, plasma creatinine and a qualitative test for urinary protein. The full blood cell count and urinary analysis should be repeated every 1–2 weeks for the first 20 weeks or at the time of each injection. Furthermore, the patients should be asked about skin itching and mouth ulcers before each injection. The 24-hour output of protein should be measured if the dip stick qualitative test for urinary protein is positive. Treatment should be ceased if the protein excretion is greater than 500 mg in 24 hours but can be restarted when urinary protein is no longer detectable.

Leflunomide

Mechanism of action

LEFLUNOMIDE is a relatively new small molecule DMARD that is active after oral dosage. Through its active metabolite, the primary mode of action of LEFLUNOMIDE is the selective and reversible inhibition of dihydroorotate dehydrogenase (Fig. 7) [80]. This enzyme provides the rate-limiting step in the pyrimidine synthesis, which is accelerated in the activated $CD4^+$ T cells that proliferate rapidly during the progression of RA. This anti-proliferative effect on activated LYMPHOCYTES is likely the key effect of LEFLUNOMIDE on the pathophysiology of RA. Additionally, LEFLUNOMIDE interferes with T cell signaling and also has broad anti-inflammatory effects. For example, the active metabolite is a potent inhibitor of NF-κB activation and causes a dose-dependent inhibition of cytokine production (including TNF). LEFLUNOMIDE also decreases the local production of synovial METALLOPROTEINASES, which suggests a mechanism by which it acts to prevent joint destruction.

Pharmacokinetics and metabolism

LEFLUNOMIDE is rapidly and almost completely converted to an active open chain metabolite by first-pass metabolism in the gut wall and liver (Fig. 7). The

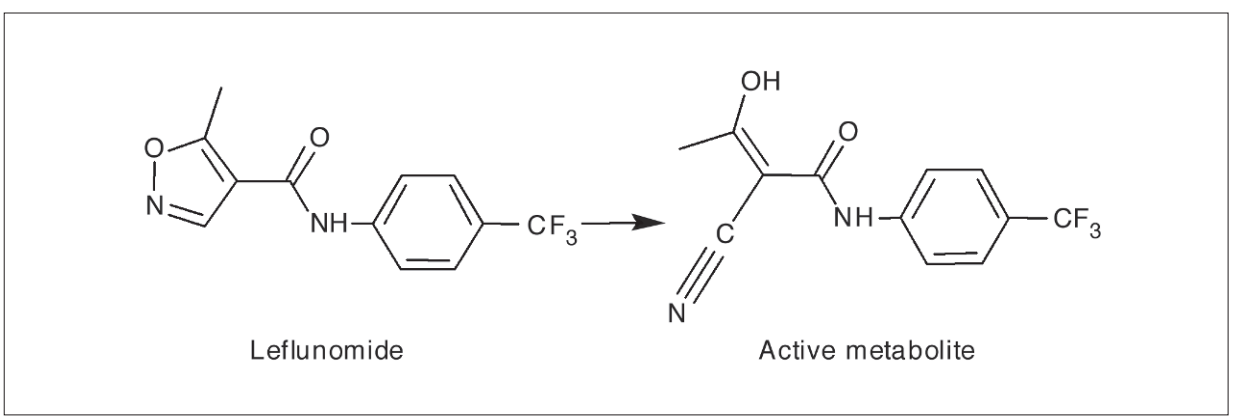

FIGURE 7. STRUCTURES OF LEFLUNOMIDE AND ITS ACTIVE METABOLITE
The active metabolite is the form that circulates in plasma and is frequently known by its code A77 1726.

active metabolite of LEFLUNOMIDE has a long $t_{1/2}$ of between 15 and 18 days because of its enterohepatic recirculation [81]. About 90% of a single dose of LEFLUNOMIDE is eliminated; about half in urine primarily as metabolites, while about 50% is secreted in bile as the active metabolite and is ultimately excreted in feces. Because the active metabolite relies heavily on biliary excretion for its CLEARANCE, and also given its risk of hepatotoxicity, LEFLUNOMIDE is contraindicated in patients with hepatic impairment.

The active metabolite of LEFLUNOMIDE may take 15–20 weeks to reach steady-state plasma concentrations. To achieve therapeutic concentrations rapidly, it is common to administer loading doses (see *Appendix*). In practice, the loading dose is often decreased or not used with the expectation of less "nuisance" problems with diarrhea or nausea, both of which may influence early PATIENT COMPLIANCE. Anecdotally, clinical EFFICACY is maintained but delayed by a few weeks.

The active metabolite binds strongly to cholestyramine within the gastrointestinal tract. The result is that its plasma $t_{1/2}$ is reduced to approximately 1 day and cholestyramine is used when rapid elimination of the active metabolite is required (see *Adverse reactions* below).

Clinical indications and efficacy

The EFFICACY of LEFLUNOMIDE has been well examined in RA. Its EFFICACY is similar to that of METHOTREXATE or SULFASALAZINE (Tab. 3). It not only decreases symptoms, and increases function and quality of life in RA, but also retards radiographic joint damage [82]. Clinical improvement has been sustained for up to 5 years. COMBINATION THERAPY with LEFLUNOMIDE and METHOTREXATE is also effective and well tolerated in patients inadequately responding to METHOTREXATE alone [83].

Apart from its value in the treatment of RA, LEFLUNOMIDE is useful in the treatment of PSORIATIC ARTHRITIS and has been used in a small cohort of SLE patients with the drug appearing to be efficacious and safe [84].

Adverse effects

The most common adverse events associated with LEFLUNOMIDE treatment are gastrointestinal; these usually decrease with time and/or dose reduction. Rash and reversible alopecia are also common.

The cytostatic effect of LEFLUNOMIDE may explain some of the side effect profile, such as reversible alopecia and conversely the lack of OPPORTUNISTIC INFECTIONS. Most memory T cells circulate in the G_0 phase, and therefore do not require dihydroorotate for any *de novo* pyrimidine synthesis, and are not susceptible to the anti-proliferative effect of LEFLUNOMIDE. In addition, because of the sparing of the salvage pathway, the replicating cells in the gastrointestinal tract and hematopoietic system are relatively unaffected, thus explaining the lack of mucositis or marrow toxicity.

When used as monotherapy in clinical trials abnormal transaminase levels are noted in 5–15% of patients, but these effects were generally mild (less than twofold elevations) and reversible, and usually resolved while continuing treatment. Post-marketing surveillance shows that almost all cases of hepatic dysfunction had other confounding factors present [85]. LEFLUNOMIDE may increase plasma levels of cholesterol and low-density lipoproteins in a progressive manner but long-term effects of this are unknown.

LEFLUNOMIDE is absolutely contraindicated in women who are or may become pregnant, because of its teratogenic effects (lymphomas) in animals. Termination of pregnancy is generally recommended if the patient has been on LEFLUNOMIDE, even though there have been a number of reported cases of delivery of full-term healthy infants [86]. Because of the prolonged $t_{1/2}$ of the active metabolite, any woman taking LEFLUNOMIDE who is contemplating pregnancy should allow the plasma concentrations of the active metabolite to fall below 0.02 mg/L. This may take several months because of its long $t_{1/2}$. Alternatively, the elimination of the active metabolite can be accelerated by cholestyramine. The active metabolite of LEFLUNOMIDE diffuses into breast milk, although it is not known if the concentrations are sufficient to cause toxicity. At this stage, however, it is contraindicated in nursing mothers.

Any suspected toxicity may be further evaluated by use of a short course (1–2 days) of cholestyramine at lower dose (4 g three times daily). This will often reverse the side effect, be it rash or diarrhea or other, quite quickly.

Monitoring

Baseline investigation should include hepatitis B and C serology and any persistent hepatic dysfunction should be further investigated. Patients should be advised to reduce alcohol consumption because of possible greater liver impairment. Liver function tests (including transaminases) should be monitored every 4–6 weeks for at least 6 months and longer if patients are taking COMBINATION THERAPY with METHOTREXATE or other hepatotoxic drugs. Thereafter, liver function tests should be repeated at least every 3 months, more frequently if transaminases have increased. Usually complete blood counts are performed at the same frequency. Alcohol should be avoided if patients are taking the combination of METHOTREXATE and LEFLUNOMIDE. All women of childbearing age should have a negative pregnancy test before beginning the drug and should be counseled to use effective forms of contraception.

Sulfasalazine

SULFASALAZINE consists of mesalazine (aminosalicylate) and a sulfonamide, sulfapyridine, linked by an azo bond (Fig. 8). This drug was synthesized in the late 1930s and originally developed and used on the basis of a belief in an infectious cause of RA [87]. The mode of action of SULFASALAZINE is unclear, although it has been reported to inhibit AICAR transformylase and, therefore, may act in a similar fashion to METHOTREXATE through the accumulation of adenosine [88]. Oddly, it is not known whether SULFASALAZINE or its metabolites are therapeutically active. The apparent antirheumatic activity of sulfapyridine indicates that it may be the active metabolite [89]. On the other hand, both SULFASALAZINE and olsalazine appear active in the treatment of ankylosing spondylitis [90], yet olsalazine is a dimer of mesalazine. Olsalazine is, like SULFASALAZINE, metabolized in the large bowel but, as is evident from its structure, olsalazine can yield only mesalazine. It is of note that SULFASALAZINE is used in both the treatment of RA and inflammatory bowel diseases. Furthermore, many patients with the bowel diseases also develop arthritic states of varying severity. These similarities indicate a commonality in the cause of the diseases and a common mode of action of SULFASALAZINE, although the reason for these parallel diseases and treatments is unknown.

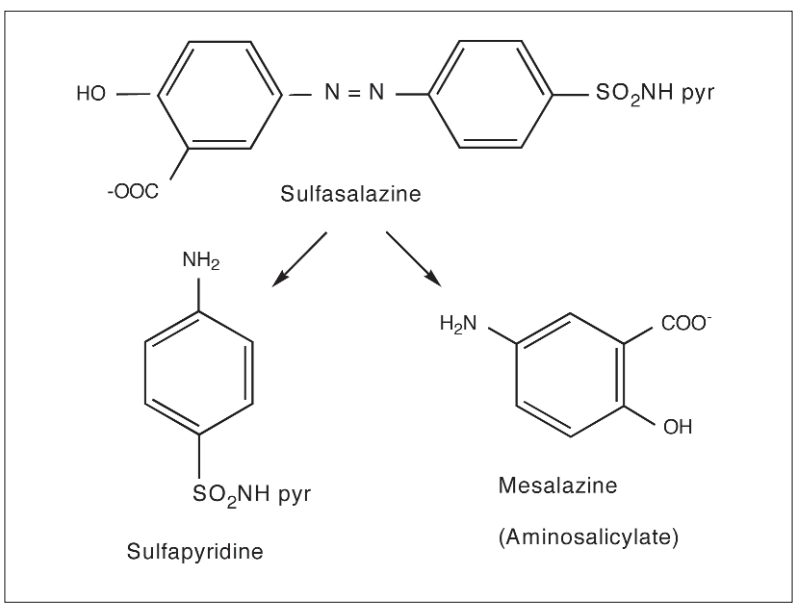

FIGURE 8. STRUCTURE AND INITIAL METABOLISM OF SULFASALAZINE
Reduction of the azo bond of sulfasalazine within the large intestine yields sulfapyridine and aminosalicylate. Sulfapyridine is subsequently absorbed while the absorption of aminosalicylate is incomplete. pyr, pyridine residue.

Metabolism and pharmacokinetics

SULFASALAZINE is administered orally, and upon reaching the large bowel, the azo bond is reduced by colonic bacteria to yield sulfapyridine and mesalazine (aminosalicylate) (Fig. 8). Sulfapyridine is absorbed, whereas mesalazine is poorly absorbed, although low concentrations of the acetylated metabolite are found in plasma. Although metabolized in the large intestine, unchanged SULFASALAZINE also achieves substantial concentrations in plasma. During daily treatment with 2 g SULFASALAZINE, the average concentrations of the unchanged drug are about 4 mg/L (10 μM) [91].

Clinical indications and efficacy

SULFASALAZINE is widely used and effective in the treatment of RA, as well as ankylosing spondylitis and HLA B27-related arthropathies. In particular, SULFASALAZINE slows the joint destruction of RA [92].

Adverse effects

Although SULFASALAZINE can produce a wide range of side effects, it is among the best, tolerated DMARD (see *Appendix*), along with hydroxychloroquine, METHOTREXATE and auranofin. Toxicity is most frequent in the first 2–3 months of usage, but its likelihood can be reduced by gradually increasing the dosage and the use of enteric coated formulations. Serious side effects are rare, and most adverse effects are eliminated if the dose is reduced from the usual 1 g twice a day. Nausea and upper abdominal discomfort are the most frequent side effects at the start of the therapy. Leukopenia is very uncommon, but can develop rapidly. Its occurrence is most likely in the first 6 months of therapy but can develop later. The metabolite, sulfapyridine, can produce hemolysis in patients with a deficiency of glucose-6-phosphate dehydrogenase. SULFASALAZINE should be avoided at around the time of conception and pregnancy, although no teratogenicity has been reported for this drug.

Monitoring

For the rapid detection of hematological side effects, it is recommended that complete blood cell count is performed before commencement of treatment. For the follow-up visits, complete blood cell counts every 2–4 weeks for the first 3 months are suggested, and at greater intervals subsequently. The hemolytic anemia in patients with a deficiency of glucose-6-phosphate dehydrogenase will be detected in blood counts. Baseline measurement of hepatic transaminases is advised in patients with known or suspected liver disease.

Corticosteroids

The general chemistry, physiology and pharmacology of the corticosteroids are discussed in chapter C13. Aspects of the clinical effects of the corticosteroids that are relevant to the treatment of RA are discussed below.

Rationale

In relation to the rheumatic diseases, the most profound effects of corticosteroid are mediated through inhibition of the expression of pro-inflammatory genes, including many of the mediators involved in the etiopathogenesis of RA. This broad spectrum of effects is mediated by the interaction between the occupied glucocorticoid RECEPTOR and the NF-κB complex, resulting in inactivation of the transcriptional effects of NF-κB [93]. Although these effects of corticosteroids are very significant, corticosteroids also induce the expression of a number of anti-inflammatory genes, including annexin I (formerly known as lipocortin 1) and MAP kinase phosphatase 1, an endogenous inhibitor of MAP kinase activation and hence of cell activity in INFLAMMATION.

Pharmacokinetics

Prednisolone, the active metabolite of prednisone, is the major corticosteroid used in the treatment of RA. The plasma $t_{1/2}$ of prednisolone is short, of the order of 2 hours, but the pharmacological effects of prednisolone, as is the case with all corticosteroids, are prolonged because of its strong binding to nuclear RECEPTORS. The change they induce in protein synthesis also develops slowly and is lost slowly. As a result, prednisolone, is usually only administered once a day.

Other corticosteroids, such as betamethasone, have also been used for the treatment of rheumatic diseases but their duration of action is generally longer with greater suppression of the pituitary-adrenal axis.

Corticosteroids are also administered by intra-articular injection. This provides a depot in the joint from which the corticosteroid dissolves slowly, thereby producing a prolonged anti-inflammatory effect in the joint. Systemic effects are seen due to absorption from the joint and may also be prolonged.

Clinical indications and efficacy

In addition to their wide use in the treatment of patients with RA, corticosteroids are used for all inflammatory arthritic states and conditions such as vasculitis and polymyalgia rheumatica. Oral or intra-articular corticosteroids significantly improve the clinical symptoms of RA and they are superior to the NSAID in this respect. The effect is rapid and most pronounced in the first weeks of administration. Patients with severe RA, vasculitis or active SLE may require high doses of oral corticosteroids, or if the patients are acutely ill, high doses of intravenous corticosteroids. Large single doses of corticosteroids (pulse therapy) have a beneficial effect for at least several weeks and may be useful while the effects of DMARD are developing [94].

The long-term use of corticosteroids in RA is controversial. A reduced rate of joint destruction has been reported but, in one study, no additional effect of low-dose prednisolone (7 mg daily) was found in patients treated with SULFASALAZINE [95]. Overall, the aims of treatment with the corticosteroids should always be to keep the dose and duration of therapy to the minimum that is compatible with good disease control.

Intra-articular corticosteroid injections are an important treatment for monoarticular inflammatory synovitis or for single joints that are difficult to control in the polyarthritic patient. The long-term effects of intra-articular therapy are unclear, with animal studies suggesting both beneficial and detrimental effects on articular cartilage. Generally, an individual joint may be injected up to 3–4 times per year, but no more in weight-bearing joints because of the risk of degeneration of cartilage.

Adverse effects

The adverse effects of the corticosteroids are outlined in chapter C13. In contrast to their anti-inflammatory effects, the side effects of corticosteroids are mostly due to the induction of genes outside the IMMUNE SYSTEM. In part for this reason, corticosteroid side effects are generally dose-related. The catabolic effect or corticosteroids on bone is strongest in the first 6–12 months after initiation of the drug. It is recommended that a dose equivalent to the physiological replacement dosage of cortisol (7.5 mg prednisolone per day) should not be exceeded, as increased OSTEOPOROSIS occurs with increasing dose [96]. Larger doses are often needed in other inflammatory rheumatic conditions such as polymyalgia rheumatica, but this limitation of dosage is often possible in the treatment of RA. To minimize the dose of corticosteroids, it is common to administer corticosteroids in conjunction with DMARD.

A major problem with the long-term use of corticosteroids is suppression of the hypothalamic-pituitary adrenal axis. This problem is reduced, but not eliminated, by administration in the morning. Alternate day administration reduces the suppressive effect further, although it is often not practicable because of inadequate clinical response. For the hypothalamic-pituitary axis to recover, systemic corticosteroids must be withdrawn slowly, particularly when daily dosage is below the equivalent of 10–15 mg prednisolone and the systemic steroid has been used for more than about 3 weeks. Dosage of the systemic corticosteroids must be increased temporarily if patients become acutely unwell or if there is evidence of adrenal insufficiency.

Combination DMARD therapies

COMBINATION THERAPY for the treatment of RA was developed because:

(i) There may also be an additive or synergistic effect from combinations. Despite the lack of knowledge on mechanisms of DMARD action, it seems likely that these drugs work in different

ways. Thus, modifying or inhibiting the disease processes at multiple sites may gain a greater degree of suppression of RA with less treatment resistance.

(ii) Combinations of DMARD may allow a decreased dose of one or both agents, leading to a concomitant decrease in adverse effects without diminished EFFICACY.

COMBINATION THERAPY can be delivered in at least three different styles. Multiple medications can be initiated together and combined dosage of DMARD maintained. Alternatively, one or more of the DMARD is gradually withdrawn. This is the "step down" approach. Alternatively the medications can be "stepped up", commencing with one agent and adding another DMARD if the desired outcome is not achieved. DMARD can be combined with bDMARD and a widely advocated combination is METHOTREXATE and an anti-TNF agent.

Table 4 shows the results from several recent trials on combinations of DMARD, including bDMARD,

TABLE 4. COMBINATION THERAPIES IN EARLY RA. *Adapted from Emery et al. [97, 98]*

Trial name	Design[1]	Therapy	Results
COMET	1 year early RA	Combination methotrexate (titrated doses) + etanercept (50 mg/week) vs Methotrexate (titrated doses)	Patients in remission: Combination 50% Methotrexate only 28%. Lack of progression of X-ray of joints: Combination 80% Methotrexate only 59%
COMET continuation	Groups split after 1 year and trial continued for further 1 year	Combination continuation vs Methotrexate step up to combination vs Methotrexate only for 1 year then step up to combination vs Combination step down to methotrexate only vs Methotrexate only continuation	Patients in remission: Combination continuation 57% Methotrexate only step up to combination 58% Combination step down to etanercept only 50% Methotrexate only continuation 35% Lack of progression of X-ray of joints: Combination continuation 90% Methotrexate only step up to combination 75% Combination step down to etanercept only 75% Methotrexate continuation 67%
TEMPO	3 years, 3 years follow-up	Etanercept + methotrexate vs etanercept vs methotrexate	Combination clinically better than either monotherapy, with less X-ray progression at follow-up if received etanercept
COBRA	28 week, step down 28 week, observed 24 week, 11 years follow-up	Prednisolone + methotrexate + sulfasalazine vs sulfasalazine, followed by step down of prednisolone, then step down of methotrexate	Rapid benefit from combination, reducing after withdrawal of prednisone and disappearing after methotrexate withdrawal – at 5 years minimal clinical difference. Reduced X-ray progression with combination to 5 years, at 11 years no difference

Table 4 *(continued)*

Trial name	Design[1]	Therapy	Results
Dougados et al.	1 year	Sulfasalazine + methotrexate vs sulfasalazine vs methotrexate	Combination not clinically superior to individual drugs
PREMIER	DB 2 years	Adalimumab + methotrexate vs adalimumab vs methotrexate	Combination better than monotherapy, with less X-ray progression
BeST	2 years, 4 years follow-up	Sequential DMARD monotherapy vs step up combination therapy vs initial combination therapy with tapered high dose prednisone, vs initial combination therapy with infliximab	Combination therapy with either prednisone or infliximab resulted in early functional benefit, but no clinical difference at 2 years. Initial combinations had less X-ray progression than sequential monotherapy or step-up combination therapy at 4 years
FIN-RACo	2-year trial, 11 years follow-up	Monotherapy with sulfasalazine (later methotrexate) ± prednisolone vs sulfasalazine + methotrexate + hydroxychloroquine and prednisolone. Treatment restrictions removed at 2 years, targeting remission continued	Initial combination therapy better with more remissions during trial. After 11 years significantly greater minimal disease activity and remission in combination group, but HAQ score similar
ASPIRE	1 year	Placebo + methotrexate vs methotrexate + infliximab (3 mg/kg and 6 mg/kg)	Combined therapy better
SWEFOT	1 year	Run in methotrexate, and if inadequate control addition of either sulfasalazine/hydroxychloroquine or infliximab	When methotrexate fails, addition of infliximab superior to conventional triple therapy, methotrexate + sulfasalazine + hydroxychloroquine
CARDERA	2 years	Methotrexate vs methotrexate + ciclosporin vs methotrexate and step down prednisolone vs triple therapy	Triple therapy initially better clinically but at 2 years no difference, lower HAQ scores with triple therapy and methotrexate/prednisolone. Triple therapy had least erosions, followed by methotrexate with either ciclosporin or prednisolone
CIMESTRA	2 years	Methotrexate vs methotrexate + ciclosporin; with intra-articular betamethasone as needed. Hydroxychloroquine added week 68 to both arms, ciclosporin tapered from week 76 to zero at week 104	Methotrexate monotherapy with intra-articular steroids gave excellent control, addition of ciclosporin did not effect DAS remission or X-ray progression, At 2 years ACR20/50 but not 70 better with combination. Higher creatinine in combination, hypertension prevalence not increased.

[1]*All trials were double blind except where noted*

in early RA. As is the case with many modern clinical trials, these trials have been very expensive with complex designs. To date, however, it is not possible to identify or advocate one combination as being the most effective. As is the case with many large-scale clinical trials, the trials of antirheumatic combinations are often referenced by acronyms of the trial designs (Tab. 4).

The COMET trial demonstrates the complex design of modern clinical trials on DMARD (Tab. 4). This trial compared the treatment with a variable dose of METHOTREXATE alone (starting at 7.5 and increasing as necessary to 20 mg per week over 8 weeks) against concurrent treatment with the variable dose of METHOTREXATE and a fixed dose of ETANERCEPT (50 mg per week) (Tab. 4) [97]. The apparent response to ETANERCEPT and METHOTREXATE may be confounded by changing doses of other antirheumatic drugs. To reduce this problem, yet make the trial clinically relevant, concurrent dosage with corticosteroids (≤10 mg prednisolone) or a single NSAID was allowed, provided that these drugs were started at least 4 weeks before commencing the trial. After 24 weeks of constant dosage of these additional drugs, reductions in the dose of prednisolone by 1 mg per week were permitted provided that prednisolone was tapered to 3 mg daily before the dosage of the NSAID was decreased. After 1 year, the combination treatment with ETANERCEPT and METHOTREXATE led to a less severe disease than was seen with METHOTREXATE alone (Tab. 4).

This trial was continued for a further year in which the groups were split (Tab. 4) [98]. Overall, the patients who continued treatment with the combination had less severe disease than the other groups. The number of patients whose joint damage had not progressed during continued treatment with the combination was remarkable (Tab. 4).

A step up trial on CICLOSPORIN and METHOTREXATE shows the necessity for pharmacokinetic examination in the study. Thus, the addition of CICLOSPORIN (2.5–5 mg/kg/day) to the maximally tolerated dose of METHOTREXATE increased the response to treatment in patients with active RA [99]. There was no difference in the reported toxicities. The basis for this improvement, however, may be more pharmacokinetic than an additive or synergistic response. Thus, CICLOSPORIN (3 mg/kg/day) produced a 26% increase in mean peak plasma concentration of METHOTREXATE and an 80% reduction in the metabolite 7-hydroxymethotrexate [100]. The metabolite is less efficacious than METHOTREXATE in rat adjuvant arthritis, and 4–17-fold less CYTOTOXIC in human cell culture. By altering the pharmacokinetic balance in favor of METHOTREXATE, the increased EFFICACY of the combination of METHOTREXATE and CICLOSPORIN can be explained.

Combining METHOTREXATE and hydroxychloroquine may produce less acute liver damage than METHOTREXATE alone. Triple therapy of METHOTREXATE, SULFASALAZINE and hydroxychloroquine, also appears useful, although it is difficult to make good comparisons of the various treatments (Tab. 4).

Current research directions

Intracellular signal pathways

TNF signaling is *via* the MAP kinase pathway, which includes p38 kinase. A number of p38α inhibitors have been developed and their trials initiated in RA, with disappointing clinical benefit offset by increased toxicity. JAK kinases are essential for lymphocyte activation as they facilitates signal transduction of for IFN, IL-2, IL-4, IL-6, IL-7, IL-9, IL-15, and IL-21 *via* binding to STAT, allowing translocation to the nucleus and initiation of transcription of TARGET genes. A JAK-3 inhibitor has achieved ACR responses similar to that obtained with anti-TNF therapy [101]. Activated by the ligation of Fc and B cell RECEPTORS, the spleen tyrosine kinase pathway (Syk) ultimately leads to MAP kinase activation and has most recently been targeted. Preliminary reports of the inhibitor, fostamatinib, suggest clinical outcomes as good if not better than anti-TNF agents, with the most common adverse event being diarrhea and NEUTROPENIA [7].

Biosimilars

The development and production of bDMARD and other biological pharmaceuticals make them highly

individual products. Biological products which are similar, but not identical to existing bDMARD, are being developed and are termed biosimilars. However, their beneficial clinical effects and toxicity may not be identical to those that are presently available, and thorough preclinical and clinical testing will be necessary before biosimilars are marketed. The biological products thus differ from the classical low molecular weight drugs, which have a defined structure and exact copies can be made.

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Immunotoxicology

Immunotoxicology

Raymond Pieters, Joseph G. Vos†, Jan Willem van der Laan and Henk van Loveren

Introduction

Pharmaceuticals for human use comprise a very wide variety of product types. These include traditional products (i.e., chemically synthesized or derived from NATURAL resources) as well as biological products (such as vaccines and blood products isolated from biological sources) and biotechnology-derived pharmaceuticals (such as peptide/protein products manufactured by RECOMBINANT DNA techniques, MONOCLONAL ANTIBODIES and GENE THERAPY products). In the interest of the public, these medicinal products are subject to worldwide regulatory control by government authorities. The major objective of this regulation is to ensure that the benefit of the products to the patients is not outweighed by their adverse effects. To achieve this goal, the authorities carefully assess the balance between EFFICACY and safety. If this balance is positive, they allow marketing. To support applications for marketing authorization, the pharmaceutical industry, therefore, has to submit scientific data which prove that their products are efficacious and acceptably safe in the proposed therapeutic indication. Furthermore, the pharmaceutical quality of the products applied for has to meet high standards.

Chemicals used for a variety of purposes can have adverse effects on the IMMUNE SYSTEM of both animals and humans. In the case of drugs, this can be the result of pharmacological interference with the IMMUNE SYSTEM, or an undesired reaction. One form of immunotoxicity is the direct toxicity of the compound to components of the IMMUNE SYSTEM, which often leads to suppressed function. This may result in decreased resistance to infection, the development of certain types of tumors or immune dysregulation and stimulation, thereby promoting ALLERGY or AUTOIMMUNITY. Other types or manifestations of immunotoxicity include ALLERGY or AUTOIMMUNITY in which the compound causes the IMMUNE SYSTEM to respond as if the compound were an antigen or to respond to self-ANTIGENS that have been altered by the chemical. A more recently recognized manifestation of immunotoxicity, called cytokine release syndrome, is due to overt release of CYTOKINES which may cause acute clinical phenomena.

Except for cancer patients on chemotherapy and organ transplant patients on long-term immunosuppressive therapy, there is little evidence that drugs are associated with undesired, clinically significant, direct IMMUNOSUPPRESSION. However, only a few valid epidemiological studies of immunologically based diseases have been carried out [1], probably due to the complication of such studies by confounding factors such as (disease-associated) stress, nutritional status, lifestyle, (co)medication and genetics. Few conventional drugs have been shown to induce unexpected enhancement of immune competence. Unwanted immunostimulation has gained attention primarily through the introduction of new biotechnologically manufactured drugs such as CYTOKINES. Drug-induced HYPERSENSITIVITY reactions and autoimmune disorders are a major concern, and often the reason for withdrawing drugs from the market or restricting their use.

For the detection of chemically induced direct immunotoxicity, animal models have been developed, and a number of these methods have been validated. Several compounds, including certain drugs, have been shown in this way to cause IMMUNOSUPPRESSION. Methods are also available for the detection of skin allergic responses, whereas no validated test is available to predict potential induction of AUTOIMMUNITY.

In this chapter, the various mechanisms of immunotoxicity by which pharmaceuticals affect different

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cell types and interfere with immune responses, ultimately leading to immunotoxicity, are introduced and discussed. Further, procedures for preclinical testing of drugs are covered, comprising direct immunotoxicity as well as sensitizing capacity. This section is followed by consideration of procedures for clinical and epidemiological testing of drugs. Finally, regulatory aspects of immunotoxicity are discussed, including current guidelines and new developments in immunotoxicity assessment.

Mechanisms of immunotoxicity by pharmaceuticals

Effects on precursor stem cells

Precursor STEM CELLS that are responsible for replenishing peripheral LEUKOCYTES reside in the BONE MARROW, making it an organ that harbors many highly proliferating cells. All leukocyte lineages originate from these STEM CELLS, but once distinct subsets of LEUKOCYTES are established, their dependence on replenishment from the BONE MARROW differs vastly. The short-lived NEUTROPHILS rely heavily on proliferation and new formation in the BONE MARROW, as each day more than 10^8 NEUTROPHILS enter and leave the circulation in a normal adult. In contrast, MACROPHAGES are long-lived and have little dependence on new formation of precursor cells [2]. The adaptive IMMUNE SYSTEM, comprising antigen-specific T and B LYMPHOCYTES, is almost completely established around puberty and therefore is essentially BONE MARROW independent in the adult.

As a consequence of their high proliferation rate, STEM CELLS in the BONE MARROW are extremely vulnerable to antiproliferative cytostatic drugs such as the antineoplastic drugs, cyclophosphamide, and METHOTREXATE, and the antirheumatic AZATHIOPRINE [1, 3] (Fig. 1). This is particularly the case at high doses of these drugs, and lineages like NEUTROPHILS, which are extremely BONE MARROW dependent, are most vulnerable and affected first by treatment with these drugs. After prolonged exposure, MACROPHAGES and T or B cells of the adaptive IMMUNE SYSTEM are also suppressed.

Effects on maturation of lymphocytes

After leaving the BONE MARROW, cells of both the T cell and the B cell lineages mature into antigen-specific LYMPHOCYTES. T LYMPHOCYTES mature in the thymus during a process referred to as thymocyte differentiation, which is a very complex selection process that takes place under the influence of the thymic microenvironment and ultimately generates an antigen-specific, host-tolerant population of mature T cells (see chapters A2 and A3). Because this process involves cellular proliferation, gene rearrangement, apoptotic cell death, RECEPTOR up- and down-regulation and antigen-presentation processes, it is very vulnerable to a number of chemicals, including pharmaceuticals (Fig. 1). Drugs may TARGET different stages of T cell differentiation: BONE MARROW precursors (AZATHIOPRINE); proliferating and differentiating thymocytes (AZATHIOPRINE); antigen-presenting thymic epithelial cells and DENDRITIC CELLS (CICLOSPORIN) [4]; cell death processes (corticosteroids) [5] (Fig. 2).

In general, immunosuppressive drugs that affect the thymus cause a depletion of peripheral T cells, particularly after prolonged treatment and during early stages of life when thymus activity is high and important in establishing a mature T cell population.

After the BONE MARROW stage, B cells mature in the spleen. With the exception of certain MONOCLONAL ANTIBODIES, there are no drugs that specifically affect B cell development, although some studies claim a more or less B cell-specific effect of cyclophosphamide and METHOTREXATE. In general, suppression of the adaptive IMMUNE SYSTEM at the ANTIBODY level is the result of an effect on T cells or their development.

Effects on initiation of immune responses

Once a mature IMMUNE SYSTEM has been established, the innate and adaptive arms of the IMMUNE SYSTEM cooperate to eliminate invading pathogens. Ideally, T cells tailor the responses to neutralize invaders with minimal damage to the host. After elimination of T cells with high AFFINITY for self-ANTIGENS in the thymus, TOLERANCE for AUTOANTIGENS is further maintained in the periphery by the two distinct signals that

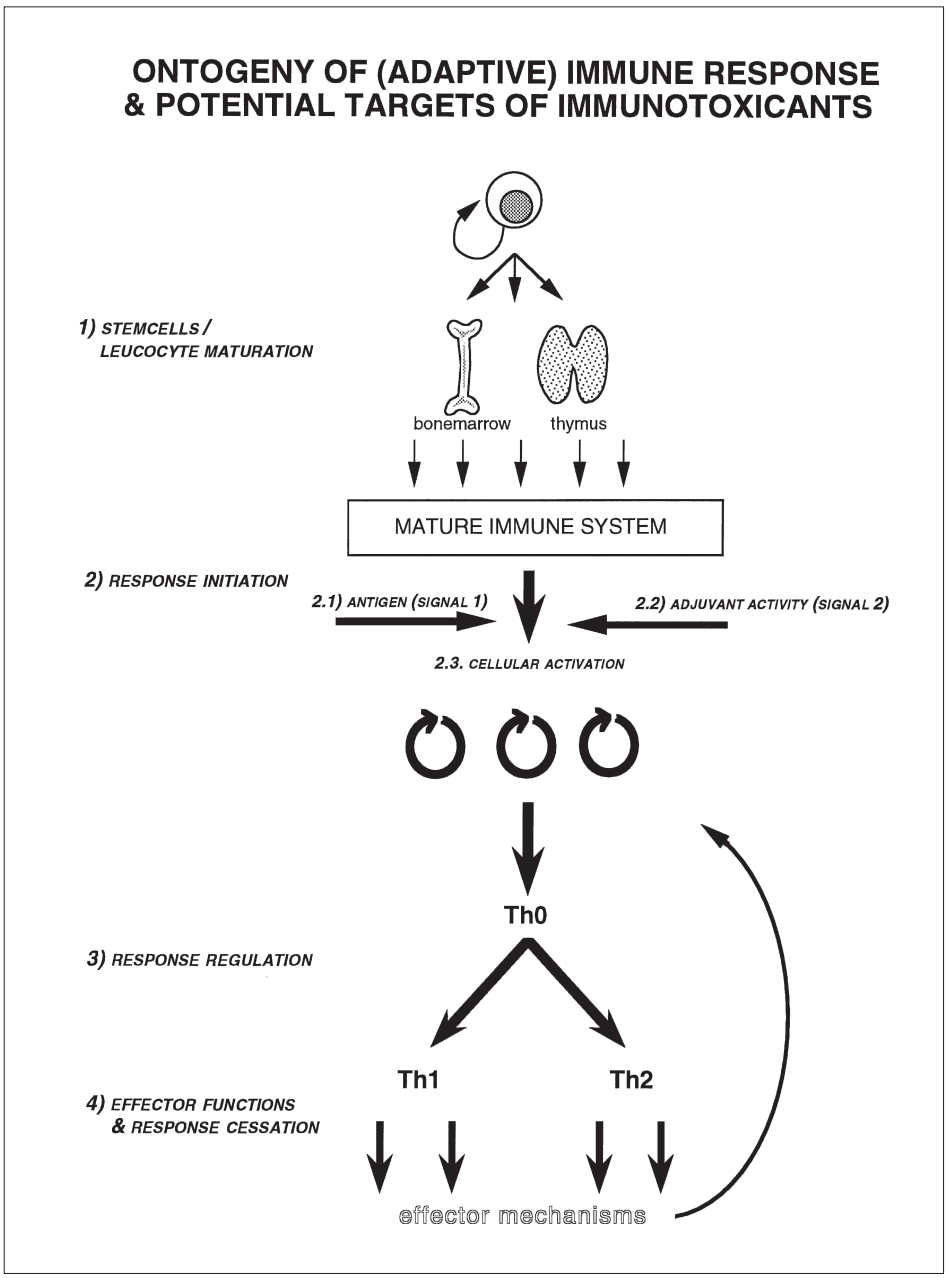

Figure 1. Ontogeny of the immune response and targets of immunotoxic pharmaceuticals

This figure represents the different steps in the ontogeny of adaptive immune responses from stem cell to response cessation. It forms our conception framework to identify potential mechanisms of immunotoxicity. Effects of pharmaceuticals on stem cells and leukocyte maturation are indicated, on the two signals that are essential for lymphocyte activation, and on the resulting cellular activation. Also indicated are the regulation and cessation of immune responses as potential targets for immunotoxic pharmaceuticals.

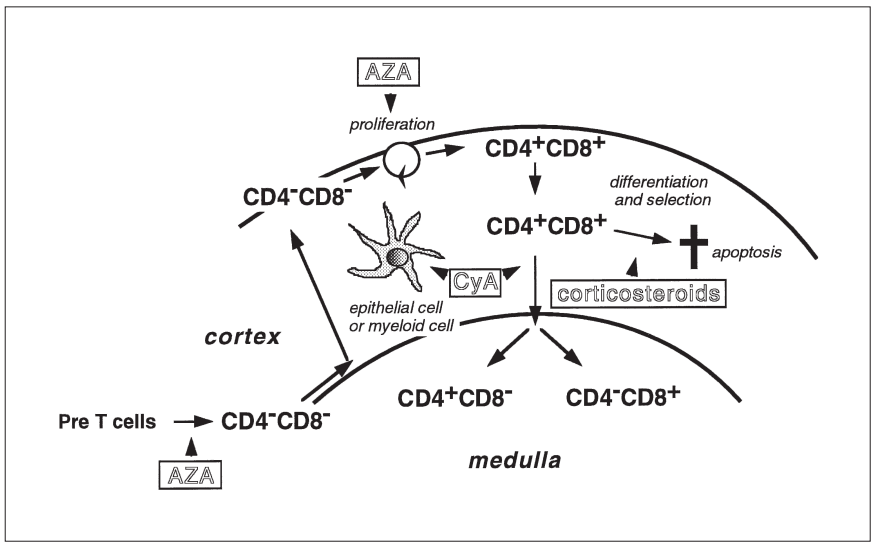

Figure 2. Schematic view of the thymus showing the cortical and medullary region
Immature pre T cells ($CD4^-CD8^-$) enter the thymus at the cortico-medullary region and migrate to the subcapsular region where they show high proliferative activity and differentiate into $CD4^+CD8^+$ thymocytes. In the cortex, most thymocytes are $CD4^+CD8^+$ and at this stage thymocytes are selected under the influence of thymic epithelial cells and are prone to apoptotic cell death. After the $CD4^+CD8^+$ stage, cells differentiate either to $CD4^+$ or $CD8^+$ cells. Stages sensitive to pharmaceutical attack are indicated: azathioprine inhibits formation of pre-T cells and may inhibit immature thymocyte proliferation; Ciclosporin interferes with thymocyte selection, possibly through an effect on thymic dendritic cells; and corticosteroids stimulate apoptosis.

govern lymphocyte activation. Signal 1 is the specific recognition of antigen *via* clonally distributed antigen RECEPTORS. Signal 2 consists of antigen-nonspecific COSTIMULATION or "help" and involves interactions of various adhesive and signaling molecules [6]. It is imperative that LYMPHOCYTES receive both signals, as antigen recognition without COSTIMULATION induces TOLERANCE, and LYMPHOCYTES are unresponsive to COSTIMULATION without an antigen-specific signal. The molecules transmitting signal 2 are thought to be expressed mainly in response to tissue damage, linking initiation of immune responses to situations of acute "danger" for the host [7]. This helps to aim immune responses at potentially dangerous microorganisms (non-self), while minimizing deleterious reactions to innocuous (non-self) ANTIGENS and to the host (self) (chapter A2).

Certain pharmaceuticals and other XENOBIOTICS can interfere with the initiation of immune responses by forming complexes with self-proteins (e.g., hapten-CARRIER complexes) or by releasing previously hidden self-ANTIGENS. By doing so, these chemicals may provide signal 1 to neo-antigen-specific T cells. But probably more chemicals may also induce conditions (e.g., cellular damage, proinflammatory conditions) that favor up-regulation of signal 2. Finally, pharmaceuticals may directly affect cellular activation following occupation of the RECEPTORS involved in the two activation signals (Fig. 1).

Interference with antigen recognition (signal 1)

Large (protein) pharmaceuticals can be antigens

Large molecular mass pharmaceuticals (>4000 Da) can function as ANTIGENS and become targets of specific immune responses themselves (reviewed in

[8]). This is particularly relevant for foreign BIOPHARMACEUTICALS, as these can activate both T and B LYMPHOCYTES. The resulting specific immune responses may lead to formation of ANTIBODIES, and induce specific memory which can lead to allergic responses to the substance. For example, passive immunization to tetanus toxin or snake venoms with serum from immunized horses causes the temporary formation of immune complexes with symptoms of fever, joint tenderness and proteinuria (serum sickness). Because serum proteins are given in large amounts and have a long half-life, SENSITIZATION and allergic reactions take place after a single dose. Similar immunotoxic effects due to immunogenicity may occur after repeated treatment with pharmaceuticals like porcine insulin, murine ANTIBODIES and biotechnologically engineered "novel proteins". In patients developing neutralizing ANTIBODIES, absence of response or reversal of clinical EFFICACY has been described [9]. The danger of immunotoxic effects due to immunogenicity is much lower when homologous RECOMBINANT human or "HUMANIZED" proteins are used as pharmaceuticals. Other factors that determine whether a certain biopharmaceutical raises an immune response are route of exposure, contaminants, and formulations [8].

Reactive pharmaceuticals can form haptens

Low molecular mass pharmaceuticals cannot function as ANTIGENS, because they cannot as such be presented by MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) molecules to T cells. Reactive drugs that bind to proteins and thus form hapten-protein complexes (also called protein-adducts) may render these proteins immunogenic. In other words, if haptenated EPITOPES derived from the protein-adducts are presented by MHC, T cells may become primed and in turn provide COSTIMULATION to specific B cells. This effect is responsible for allergic responses to many new (neo)EPITOPES formed by chemical HAPTENS, including pharmaceuticals, occupational contact sensitizers and respiratory sensitizers (Fig. 3). Other compounds require metabolic activation to form reactive metabolites that bind to proteins. The anesthetic halothane, for instance, is metabolized to alkyl halides by cytochrome P450 in the liver. The alkyl halides bind to microsomal proteins including P450, and the bound HAPTENS induce an immune response that causes so-called halothane hepatitis. Other compounds can be activated by extrahepatic metabolism, in particular by the myeloperoxidase system in phagocytic cells. For instance, activated MACROPHAGES and GRANULOCYTES can metabolize the antiarrhythmic procainamide to reactive metabolites that can bind to proteins, and immune responses to these HAPTENS are considered to be responsible for the initiation of procainamide-related agranulocytosis and lupus [10].

Induction of cross-reactivity by pharmaceuticals

Formation of neo-antigenic structures, in particular covalent or non-covalent hapten-CARRIER complexes may cause stimulation of cross-reactive T cells. Cross-reactivity implies that T cells recognize not only the best fitting MHC-(neo-)peptide complex but also other structurally less related (neo-)peptides, even in the groove of unrelated MHC molecules (also termed allo-reactivity) (reviewed in [11]).

It has been found that certain drug-reactive T cell CLONES from patients were MHC-allele unrestricted and at the same time highly drug-specific, i.e., they did not respond to drug derivatives with small chemical alterations. Other drug-induced T cell CLONES appeared less stringent with respect to the structure of the drug they recognized but they were highly MHC-allele restricted. Still other drug-induced T cells responded to MHC-peptide complexes in the absence of the initiating drug, and in a MHC-allele unrestricted manner. So the SPECIFICITY of drug-reactive T cells may range from highly drug-specific and non-MHC-restricted to highly MHC-restricted and non-drug-specific. From these findings it can be inferred that drug-induced T cells may also react with AUTOANTIGENS through cross-reactivity.

Responses to haptens can spread to autoreactive responses

Chemical modification of AUTOANTIGENS can also lead to autoreactive responses to unmodified self-EPITOPES by a mechanism unrelated to cross-reactivity. ANTIGENS composed of neo- and self-EPITOPES (i.e.,

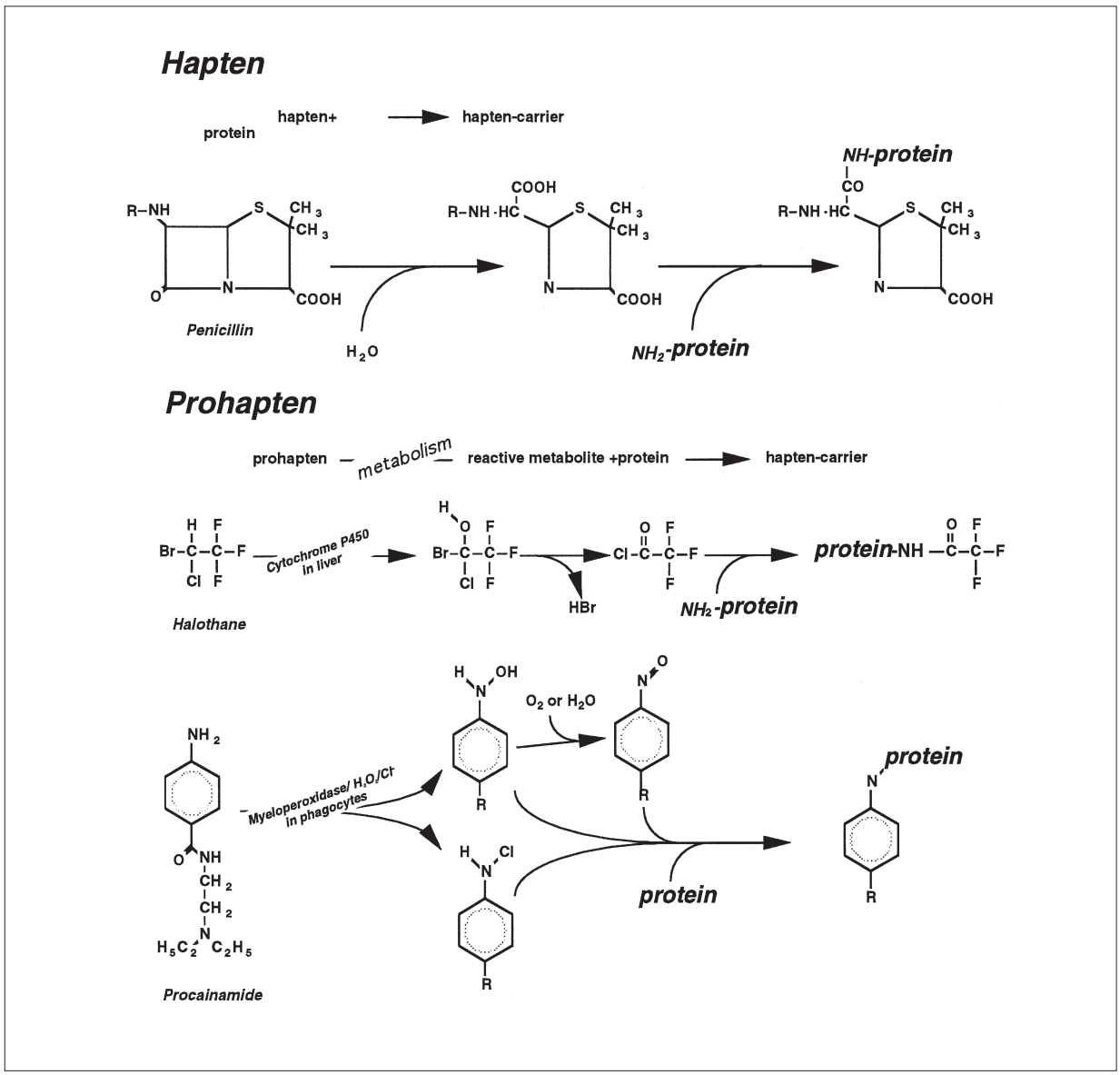

Figure 3. Haptens and prohaptens

Pharmaceuticals that are too small to attract a T cell response can become antigenic when they bind as hapten to a protein carrier. In this case, T cells responding to chemically induced neo-epitopes on the carrier provide costimulation for B cells responding to the hapten. Prohaptens require metabolic activation to a reactive metabolite that can function as a hapten. Penicillin is a well known example of a pharmaceutical that can form haptens by direct binding to proteins. In contrast, halothane itself does not form haptens, but cytochrome P450-mediated metabolism in the liver results in reactive metabolites that do bind to proteins. Procainamide can be metabolized by the myeloperoxidase/ H_2O_2/Cl^- system of phagocytes. These metabolites are very reactive and can bind covalently to nucleophilic thiol and amino groups of proteins.

haptenated AUTOANTIGENS) can be recognized and internalized by B cells specific to either the hapten or to unmodified B cell EPITOPES on the autoantigen. These cells subsequently present a mixture of neo- and self-EPITOPES complexed to distinct MHC class II molecules on their surface (Fig. 4). Since T cell TOLERANCE is obviously not established for the NEO-EPITOPES, neo-specific Th cells provide signal 2 for the B cell. This leads to production of either anti-hapten or anti-self ANTIBODIES depending on the exact SPECIFICITY of the B cell. Moreover, once these B cells are activated, they can stimulate autoreactive Th cells that recognize unmodified self-EPITOPES. The underlying process is called epitope (determinant) spreading and causes the diversification of adaptive immune responses. Responses induced by injection of mercury salts, for instance, are initially directed only to unidentified chemically created NEO-EPITOPES, but after 3–4 weeks include reactivity to unmodified self-EPITOPES [12]. The distinction between allergic and autoimmune responses induced by HAPTENS may therefore only be gradual, reflecting the relative antigenicity of the neo- and self-EPITOPES involved [13].

Pharmaceuticals can expose (epitopes of) autoantigens

Induction of self-TOLERANCE involves specific recognition of autoantigen leading to selective inactivation of autoreactive LYMPHOCYTES, and TOLERANCE is therefore not established for (EPITOPES of) AUTOANTIGENS that are normally not available for immune recognition. Pharmaceuticals can expose such sequestered (EPITOPES of) AUTOANTIGENS by disrupting barriers between the antigen and the IMMUNE SYSTEM (i.e., blood-brain barrier, blood-testis barrier, cell membranes). Tissue damage, cell death and protein denaturation induced or enhanced by pharmaceuticals

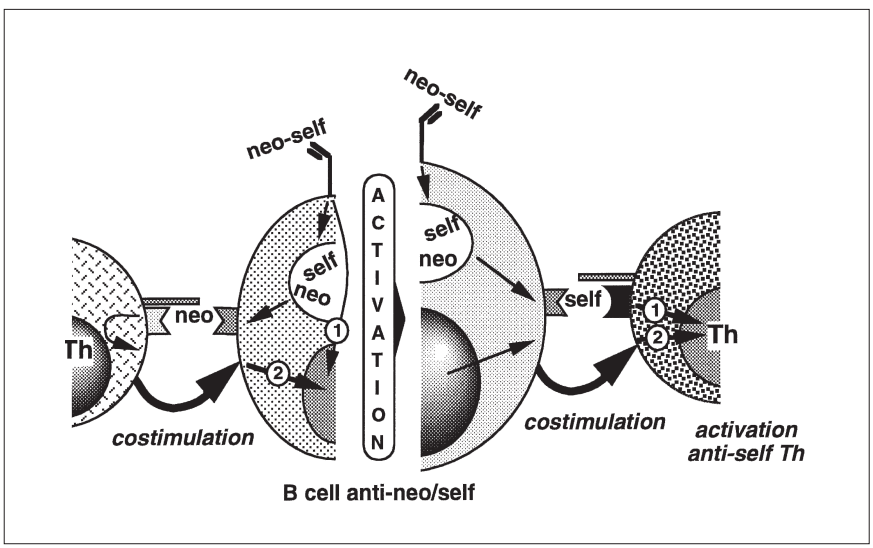

FIGURE 4. DETERMINANT SPREADING

Immune responses to haptens can spread to autoreactive responses to the carrier protein. Haptenated autoantigens are recognized and internalized by specific B cells. After uptake and processing the B cells present a mixture of the neo- and self-epitopes complexed to distinct MHC class II molecules. Naïve T cells do not respond to the self-epitopes, but neo-specific T cells provide costimulation for such B cells. This leads to activation of the B cell and production of antibodies. Moreover, when the B cells are activated, they can provide costimulation for naïve T cells that recognize unmodified self-epitopes, leading to their activation and breaking of T cell tolerance. 1: the antigen-specific or first signal; 2: the costimulatory or second signal; neo: neo-epitope.

can largely increase the availability of such (EPITOPES of) AUTOANTIGENS for immune recognition (Fig. 5). Moreover, altered antigen processing, augmenting the presentation of previously undisclosed EPITOPES, increases the availability of these so-called subdominant or CRYPTIC EPITOPES for recognition by T cells [14, 15]. It has been shown, for instance, in mouse studies that antigen preincubated with Au(III), the oxidized metabolite of the antirheumatic auranofin, elicits additional T cell responses to CRYPTIC EPITOPES [13].

Interference with costimulation (signal 2)

It is important to stress that antigen recognition by itself does not lead to activation of LYMPHOCY-

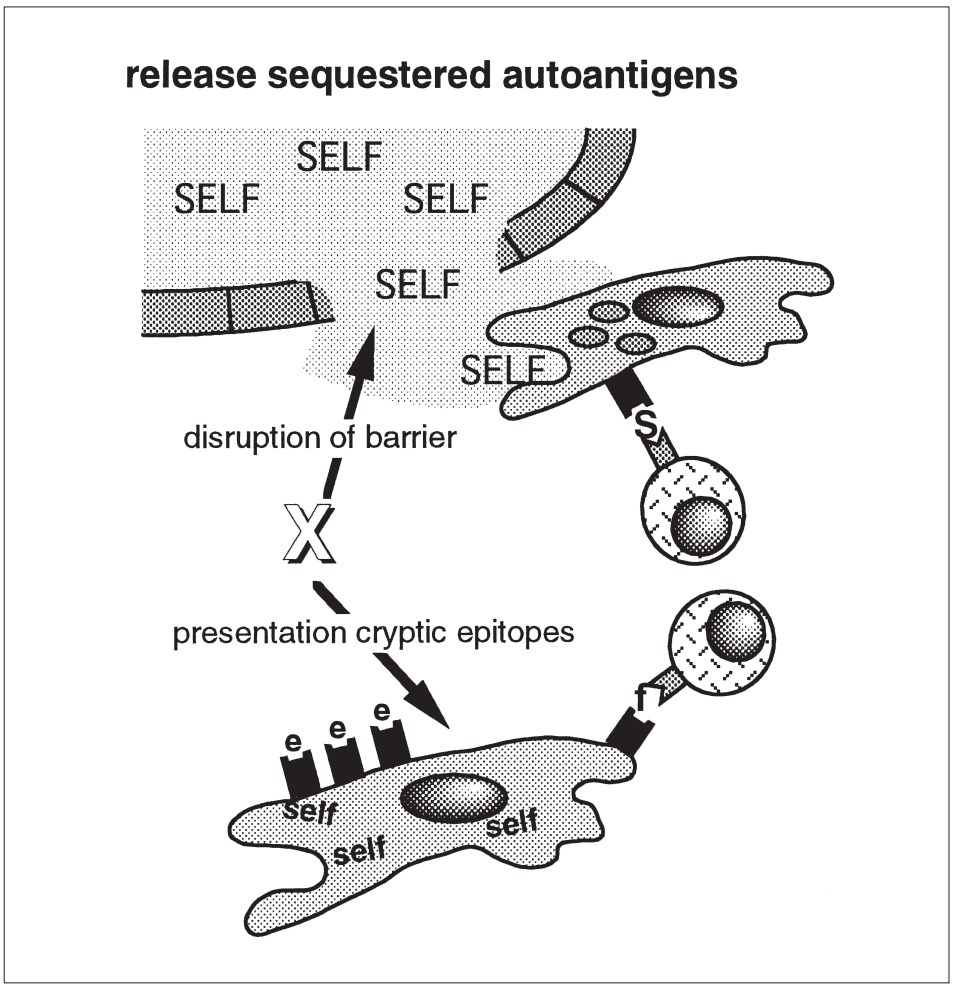

FIGURE 5. RELEASE OF SEQUESTERED SELF-EPITOPES

Pharmaceuticals can expose previously sequestered (epitopes of) self antigens by disrupting barriers between the antigen and the immune system (i.e., blood-brain barrier, blood-testis barrier, cell membranes). Similarly, augmented presentation of cryptic epitopes, as a result of altered antigen processing, increases the availability of these epitopes for recognition by T cells.

TES (above and chapter A2), but that COSTIMULATION (i.e., signal 2) is required for the initiation of immune responses [16–18]. Many XENOBIOTICS have the inherent capacity to induce this COSTIMULATION; they have intrinsic adjuvant activity. For instance, immunostimulatory responses in mice induced by d-PENICILLAMINE and phenytoin could be inhibited by blocking costimulatory interactions (i.e., mediated by CD40-CD154) with a specific monoclonal ANTIBODY to CD154 [19]. The underlying mechanisms are not always understood, but several mutually non-exclusive possibilities have been described.

Induction of inflammation

CYTOTOXIC pharmaceuticals or their reactive metabolites can induce tissue damage which leads to accumulation of tissue debris, release of proinflammatory CYTOKINES like TUMOR NECROSIS FACTOR-α (TNF-α), INTERLEUKIN-1 (IL-1), and IL-6, and attracts inflammatory cells like GRANULOCYTES and MACROPHAGES. CYTOKINES produced during this inflammatory response activate antigen-presenting cells. These present selected EPITOPES of ANTIGENS from the debris, and provide COSTIMULATION for Th cells, which leads to the initiation of an adaptive immune response [16]. Conceivably, all reactive and CYTOTOXIC pharmaceuticals can have this effect to some extent. Side effects reported after the therapeutic use of CYTOKINES have provided evidence that activation of the immune response may sometimes have deleterious consequences, such as flu-like reactions, vascular leak syndrome and cytokine release syndrome. Cytokine-induced exacerbations of underlying autoimmune or inflammatory diseases may be other complications of concern [9]. The occurrence of cytokine release syndrome has also been reported as a serious consequence of the administration of certain therapeutic MONOCLONAL ANTIBODIES [20].

Non-cognate T-B cooperation

Reactive XENOBIOTICS may also stimulate adaptive immune responses by disturbing the normal cooperation between Th and B cells. Normally, B cells receive COSTIMULATION from Th cells that cognately recognize (EPITOPES of) the same antigen. As such, B cell TOLERANCE for AUTOANTIGENS is a corollary of the T cell TOLERANCE for such ANTIGENS. However, when Th cells respond to non-self EPITOPES on B cells, such B cells may be non-cognately stimulated by the Th cell. This occurs during graft-*versus*-host responses following BONE MARROW transplantation, when Th cells of the host recognize non-self EPITOPES on B cells of the graft and *vice versa*. This leads to T and B cell activation and results in production of AUTOANTIBODIES to distinct AUTOANTIGENS like DNA, nucleoli, nuclear proteins, ERYTHROCYTES and basal membranes. Drug-related lupus is characterized by a similar spectrum of AUTOANTIBODIES, and it has therefore been suggested that non-cognate (graft-*versus*-host-like) T–B cooperation caused by T cell reactivity to HAPTENS on (autoreactive) B cells is one of the underlying mechanisms (Fig. 6) [21]. Chlorpromazine, hydralazine, phenytoin, isoniazid, α-methyldopa and procainamide are just a few of the pharmaceuticals that are associated with drug-related lupus [22].

Interference with cellular activation

Occupation of various lymphocyte RECEPTORS results in a cascade of molecular processes that eventually lead to production of GROWTH FACTORS and cellular proliferation and/or activation. Its complexity makes this cascade vulnerable to pharmaceuticals at numerous stages, although most of these chemicals TARGET very crucial processes like purine metabolism, as in the case of AZATHIOPRINE. Most drugs that interfere with cellular activation are not cell-type selective at higher doses, as all living cells depend on the same basic molecular processes. At low doses, however, drugs like AZATHIOPRINE, cyclophosphamide and METHOTREXATE appear to have a more selective effect. Azathioprine, for instance, is claimed to selectively suppress T lymphocyte function at low doses, whereas cyclophosphamide or METHOTREXATE preferentially affect B cells [1].

Suppressive effects of CICLOSPORIN and FK506, both interfering with the activation of the T cell-specific transcription factor NF-ATc, and rapamycin, preventing IL-2 RECEPTOR activation, are obviously more specific to the T lymphocyte.

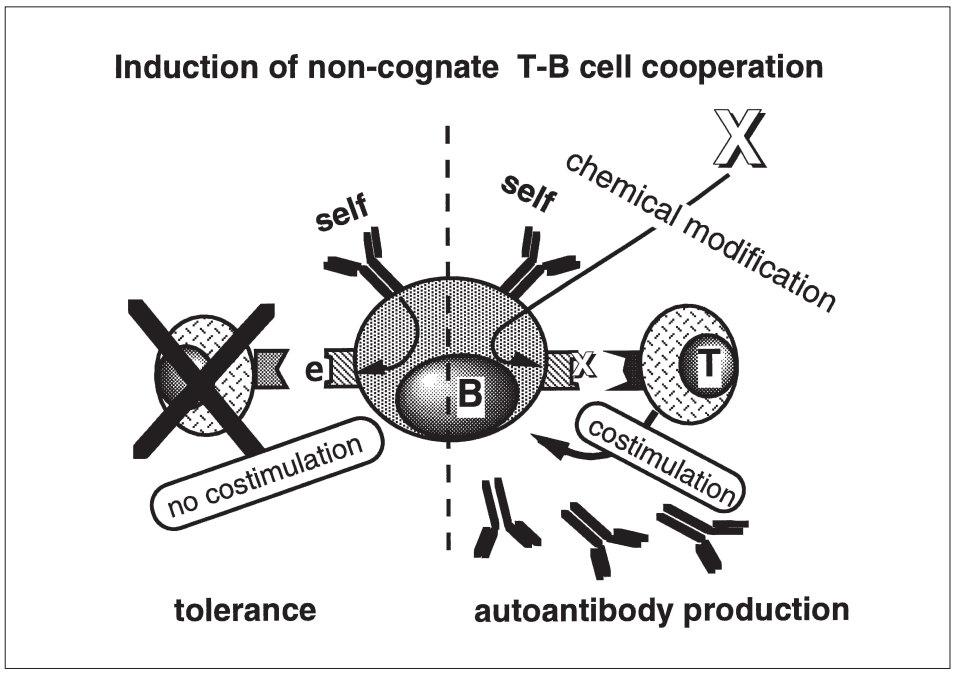

Figure 6. Non-cognate T-B cell cooperation

*Normally, activation of B cells requires costimulation from activated T cells that cognately recognize epitopes of the same antigen. B cells with specificity for autoantigens do not receive this signal because T cells have learned to ignore autoantigens. However, certain pharmaceuticals can bind to B cell proteins and Th cells activated by the neo-epitopes thus created can non-cognately provide costimulation for the (e-specific) B cell (e=a part of self for which tolerance exists). This can bypass tolerance in the T cell compartment and can lead to activation of autoreactive B cells and production of autoantibodies. This resembles stimulation of host B cells by graft T cells responding to the MHC molecules on the B cells during graft-*versus*-host reactions, and leads to a similar spectrum of autoantibodies.*

Cytokine release syndrome

In 2006, six healthy young male volunteers were enrolled in a first-in-human clinical trial with TGN1412, a new anti-CD28 monoclonal ANTIBODY that was expected to specifically stimulate regulatory T cells but appeared to directly and polyclonally stimulate T cells. The serious life-threatening symptoms that were observed were summarized as a cytokine storm [23]. Earlier cases of cytokine release syndrome were observed with muromomab (anti-CD3/Orthoclone OKT3, [24]) and alemtuzumab (Campath-1H, [25]). The mechanism behind this induction of cytokine release might be different among the various MONOCLONAL ANTIBODIES. It is a challenge for the usually small companies developing new concepts in ANTIBODY-based therapeutic research to predict this type of adverse effect. Stebbings et al. [26] has developed an *in vitro* screening method based on cytokine responses to immobilized TGN1412 of human PBMC. Recently, a workshop held at the European Medicines Agency (EMA) has discussed this approach and has indicated that the state-of-the-art in cytokine release assays is insufficient to recommend a specific assay in this respect. Assays should be used rather as a hazard identification tool, followed by adequate risk mitigation strategies (e.g., use of corticosteroids) and should not be a reason to discontinue product development [27].

Regulation of the immune response

Ongoing immune responses have to be carefully regulated to mount the most suited defense (Fig. 1). Elimination of (intra)cellular targets, like virally infected or neoplastic cells, is most efficient by Th1-driven cellular responses using CYTOTOXIC T cells and MACROPHAGES as effector mechanisms. Soluble targets, like extracellular bacteria and proteins, on the other hand, are most effectively eliminated by Th2-driven humoral responses, which rely on the formation of specific ANTIBODIES. The regulation of the type of immune response elicited and of the effector mechanisms activated is the result of a complex interplay of CYTOKINES produced by MACROPHAGES, DENDRITIC CELLS, MAST CELLS, GRANULOCYTES and LYMPHOCYTES (see chapter A5) and is influenced by a number of endo- and exogenous factors. Genetic make-up, in particular genes encoding for MHC molecules, but also gender (estrogens) are among the endogenous factors, whereas the type and dose of antigen, the route of exposure but also the type of (ongoing) costimulatory adjuvant activity are among the exogenous factors [28]. The role of the genetic makeup is illustrated by the strain-dependent effects of $HgCl_2$ in small laboratory animals. This chemical is capable of inducing a Th1-dependent immunosuppressive state in an $H2^d$ strain of mice or an $RT1^l$ strain of rat, whereas it induces an autoreactive Th2-dependent response in an $H2^s$ strain of mice or $RT1^n$ strain of rat [29, 30]. However, in some cases the outcome of the response may also depend on the chemical, and not so much on the strain. For instance: $HgCl_2$ induces a Th2-like response in BALB/c mice, whereas the diabetogenic antitumor compound, streptozotocin (STZ), induces a TH1 RESPONSE in the same mouse strain [31]. Other examples are the adjuvants, complete Freund's adjuvant (CFA) and alum, which stimulate the formation of immunoglobulin (Ig) G2a and IgG1/IgG2a ISOTYPES of ANTIBODIES, respectively [28].

How chemicals exactly modulate the immune response is largely unknown, but modulation of epitope selection by MHC molecules, selective activation of the INNATE IMMUNE SYSTEM (e.g., MAST CELLS in the case of $HgCl_2$) and chemical-specific factors (e.g., macrophage activation by STZ), as well as interaction with other environmental factors (microbial influences) may all contribute to the ultimate immunotoxicological effect.

Apart from regulation of the type of immune response, the occurrence of an immune response *per se* is tightly controlled. Recently, a number of regulatory T cell subsets have been identified that suppress auto-aggressive responses and ALLERGY [32]. Studies indicate that regulatory cells are also involved in TOLERANCE to orally encountered XENOBIOTICS. For instance, it has been demonstrated that regulatory $CD4^+CD25^+FOXP3^+$ T cells prevent autoantibody production by procainamide, gold sodium thiomalate or $HgCl_2$ in mice [33]. Similarly, induction of TOLERANCE to adverse immune effects by d-PENICILLAMINE was at least partly mediated by other T cells as well, possibly interferon (IFN)-γ-producing $CD8^+$ T cells [34].

Effector functions and response cessation

To avoid unnecessary damage, the IMMUNE SYSTEM has several feedback mechanisms to stop ineffective and obsolete responses (Fig. 1). The simplest feedback is the antigen itself, as complete degradation of the response-inducing antigen usually leads to response cessation. Pharmaceuticals that impair the activity of effector mechanisms delay antigen degradation and lead to accumulation of debris. It has been demonstrated, for instance, that several drugs, including D-PENICILLAMINE and procainamide, inhibit complement factor C4. This hampers the CLEARANCE of immune complexes and may therefore lead to their deposition and excessive tissue damage (reviewed in [35]).

Some drugs may also directly stimulate effector mechanisms, such as the COMPLEMENT SYSTEM (contrast media) or EFFECTOR CELLS, such as MAST CELLS and BASOPHILS. For instance the antibiotic, vancomycin, may induce HISTAMINE release and some non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, may directly modulate the arachidonic acid pathway. In any case, anaphylactoid clinical effects may occur without involvement of specific immune recognition. For those cases, in which effector mechanisms are directly altered by drugs and clinically apparent allergic symptoms are seen, the term pseudo-ALLERGY is used (reviewed in [36]).

Clinical consequences of immunotoxicity of pharmaceuticals

In general, pharmaceuticals that inhibit cellular replication or activation induce IMMUNOSUPPRESSION that is dose dependent. Particularly, impaired activity of the first line of defense formed by the NATURAL IMMUNE SYSTEM can have disastrous consequences. These are generally not influenced by the genetic predisposition of the exposed individual, but an actual outbreak of (opportunistic) infections or increased frequency of neoplasms may depend on the general immune status prior to exposition. This explains why immunosuppressive pharmaceuticals are most likely to have clinical consequences in immunocompromised individuals such as young children, the elderly and transplant recipients.

Immunotoxic pharmaceuticals that somehow activate the IMMUNE SYSTEM can lead to autoimmune or allergic diseases. Although on a drug-by-drug basis, immune-mediated adverse effects are mostly rare, overall it is estimated that 6–10% of all adverse drug reactions are immune-mediated, and these immune-mediated adverse effects are the most frequent cause of failure during clinical development [37]. Drug-induced ALLERGY and autoimmune phenomena may have very serious clinical outcomes, such as acute liver diseases (also designated, drug-induced liver injury, DILI), BLOOD DYSCRASIAS and skin diseases. But in fact, all organs can be subject to clinical consequences induced by drugs, in which case a multi-organ syndrome can be seen. In these cases, autoimmune phenomena may be initiated by drug-specific reactions [38].

Actual development of clinical symptoms is influenced by the route and duration of exposure, the dosage of the pharmaceutical, and by immunogenetic (MHC haplotype, Th1-type *versus* Th2-type responders) and pharmacogenetic (acetylator phenotype, sulfoxidizer, aryl hydrocarbon RECEPTOR, etc.) predisposition of the exposed individual. From an immunological point of view, it is clear that the polymorphic MHC molecules select the EPITOPES that are presented to T cells, and therefore influence all immune responses, including allergic and autoimmune responses induced by pharmaceuticals. Moreover, atopic individuals that tend to mount Th2 immune responses are more susceptible to ANAPHYLAXIS triggered by an IgE response to chemical HAPTENS than typical Th1 responders. Genetic variation in metabolism of pharmaceuticals is important as it determines the formation and CLEARANCE of immunotoxic metabolites. The slow acetylating phenotype for instance, predisposes to drug-related lupus because reactive intermediates of phase I metabolism have an increased opportunity to bind proteins as they are only slowly conjugated.

Drugs known to interfere with the IMMUNE SYSTEM are normally prescribed by well-trained physicians and are taken under more or less controlled conditions. As a result, adverse effects should be recognized as soon as they become apparent and measures can be taken before permanent harm is done. However, in the case of allergenic drugs, the IMMUNE SYSTEM is sensitized, which may hamper future treatments with the same or a structurally related chemical (penicillin). In the case of strong immunosuppressant drugs, used for instance to prevent ALLOGRAFT transplant rejection or as anti-inflammatory treatment, an immunocompromised state is taken for granted. However, prolonged and severe IMMUNOSUPPRESSION may cause permanent detrimental effects when (other) neoplasms are formed, or when (opportunistic) infections occur. In the latter case, precautions (e.g., anti-microbial compounds) have to be taken. In the case of mild immunosuppressant drugs, more subtle changes in resistance to infections, such as influenza or common cold, may occur.

As the clinical consequences of exposure to immunotoxic pharmaceuticals range from immunodepressed conditions, on the one hand, to allergic and AUTOIMMUNE DISEASES on the other, preclinical testing of pharmaceuticals in laboratory animals requires different approaches. In the following sections, procedures are covered that comprise direct immunotoxicity as well as sensitizing capacity testing.

Procedures for preclinical testing of direct immunotoxicity

Testing in rodents by tiered approach

Several laboratories have developed and validated a variety of methods to determine the effects of chemi-

cals on the IMMUNE SYSTEM of rats and mice [39]. Most employ a tier-testing system, whereas some investigators have advocated multiple testing in a single animal. The tier-testing approaches are similar in design, in that the first tier is a screen for immunotoxicity with the second tier consisting of more specific or confirmatory studies, host resistance studies or in-depth mechanistic studies. At present, most information regarding these models comes from the model developed at the National Institute for Public Health and the Environment (RIVM) in Bilthoven, the Netherlands, and the model developed at the U.S. National Institute of Environmental Health Sciences National Toxicology Program (NIEHS-NTP). The RIVM tiered system [40,41] is based on the guideline 407 of the Organization for Economic Co-operation and Development (OECD), and performed in the rat using at least three dose levels, i.e., one resulting in overt toxicity, one aimed at producing no toxicity and one intermediate level. There is no immunization or challenge with an infectious agent. The first tier comprises general parameters, including conventional hematology, serum Ig concentrations, BONE MARROW cellularity, weight and histology of LYMPHOID ORGANS [thymus, spleen, lymph nodes, MUCOSA-ASSOCIATED LYMPHOID TISSUE (MALT)], flow cytometric analysis of spleen cells and possibly IMMUNOPHENOTYPING of tissue sections (Tab. 1). This approach has been used for the immunotoxic evaluation of pesticides [42] and pharmaceuticals [43].

The OECD guideline 407 includes the weight of spleen and thymus, and histopathology of these organs, in addition to lymph nodes, PEYER'S PATCHES and BONE MARROW [44]. However, it should be borne in mind that in this guideline 407, some immunotoxic compounds may not be identified as such [45]. For instance, the opiate analgesic buprenorphine [46], and the long-acting β2-adrenergic RECEPTOR agonist salmeterol [47], affect serum Ig in rats. Results of an interlaboratory validation study in the rat, using CICLOSPORIN and hexachlorobenzene, showed the importance of updating the 407 protocol with lymphoid organ weights and serum Ig levels. The study concluded that histomorphological examination of lymphoid tissues resulted in the most reliable and sensitive data to be considered in regulatory toxicology and risk assessment [48].

In this OECD guideline 407 for testing toxicants, the IMMUNE SYSTEM is not evaluated functionally. The inclusion of an *in vivo* antigen challenge test, e.g., with sheep red blood cells (SRBC), is currently considered to improve the SENSITIVITY of the toxicity test. Experimental results [49] indicate that intravenous injection with SRBC during a 30- and 90-day toxicity study did not alter hematological and clinical chemistry parameters. With the expected exception of the spleen, administration of SRBC did not significantly alter the weights or morphology of routinely analyzed tissues.

It should be noted that the array of tests currently included in the updated OECD guideline 407 is aimed at detecting potential immunotoxicity. Once immunotoxicity has been identified, further testing is required to confirm and extend the earlier findings. Further testing should include immune function testing (Tab. 1). In addition to confirming functional implications of the immunotoxicity identified, functional tests will likely provide information on no-adverse-effect levels, and are therefore valuable for the process of risk assessment. Caution is needed in determining the relevance of slight effects on immune parameters in view of the functional reserve capacity of the IMMUNE SYSTEM. In those cases, infection models can be very helpful for risk assessment, as they are tools to elucidate the actual consequences of disturbances of immune function; effects observed using such infection models have surpassed the reserve capacity of the IMMUNE SYSTEM. The fate of the pathogen and the associated host pathology, may serve as indicators of the health implications of the immunotoxicity of the test chemical. Pathogens used in these host resistance models are chosen so that they are good models for human disease [41]. With some compounds, induction of immunotoxicity occurs especially during prenatal exposure. However, so far, there are no immune parameters included in current OECD guidelines for developmental or reproductive toxicity testing [50].

The U.S. NTP has developed a tiered approach in mice that is linked closely to the standard protocol for chronic oral toxicity and carcinogenicity studies [51]. Routinely, exposure periods of 14–30 days have been used at dose levels that have no effect on body weight or other toxicological endpoints. In this

Table 1. Panel of the Dutch National Institute for Public Health and the Environment for detecting immunotoxic alterations in the rat

Parameters	Procedures
TIER 1	
Non-functional	Routine hematology, including differential cell counting Serum IgM, IgG, IgA, and IgE determination Lymphoid organ weights (thymus, spleen, local and distant lymph nodes) Histopathology of thymus, spleen, lymph nodes and mucosa-associated lymphoid tissue Bone marrow cellularity Analysis of lymphocyte subpopulations in spleen by flow cytometry
TIER 2 PANEL	
Cell-mediated immunity	Sensitization to T cell dependent antigens (e.g. ovalalbumin, tuberculin, *Listeria*), and skin test challenge Lymphoproliferative responses to specific antigens (*Listeria*); mitogen responses (ConA, PHA)
Humoral immunity	Serum titration of IgM, IgG, IgA, IgE responses to T-dependent antigens (ovalbumin, tetanus toxoid, *Trichinella spiralis*, sheep red blood cells) with ELISA Serum titration of T cell-independent IgM response to LPS with ELISA Mitogen response to LPS
Macrophage function	*In vitro* phagocytosis and killing of *Listeria monocytogenes* by adherent spleen and peritoneal cells Cytolysis of YAC-1 lymphoma cells by adherent spleen and peritoneal cells
NK cell function	Cytolysis of YAC-1 lymphoma cells by nonadherent spleen and peritoneal cells
Host-resistance	*Trichinella spiralis* challenge (muscle larvae counts and worm expulsion) *Listeria monocytogenes* challenge (spleen clearance) Rat cytomegalovirus challenge (clearance from salivary gland) Endotoxin hypersensitivity Autoimmune models (adjuvant arthritis, experimental allergic encephalomyelitis)

way, compounds are identified for which the IMMUNE SYSTEM represents the most sensitive TARGET organ system. Tier 1 includes conventional hematology, lymphoid organ weight, cellularity and histology of the spleen, thymus and lymph nodes, ex vivo splenic IgM ANTIBODY plaque-forming cell assay following SRBC immunization, *in vitro* lymphocyte proliferation after stimulation with mitogens and allogeneic cells, and an *in vitro* assay for NATURAL killer (NK) cell activity. In an adapted form of this approach, 51 different chemicals were evaluated, selected on the basis of structural relationships with previously identified immunotoxic chemicals [52]. The splenic SRBC IgM plaque-forming cell response and cell surface marker analyses showed the highest accuracy for identification of potential immunotoxicity.

Recently, the interlaboratory reproducibility of extended histopathology was studied by evaluating thymus, spleen and mesenteric lymph node of these past NTP studies, performed in the mouse using 10 chemicals and 3 positive controls [53]. The consistency was examined between four experienced toxicological pathologists with varied expertise in immunohistopathology. Agreement between pathologists was highest in the thymus, in particular when evaluating thymus cortical cellularity, good

in spleen follicular cellularity and in spleen and lymph node GERMINAL CENTER development, and poorest in spleen red pulp changes. The ability to identify histopathological changes in lymphoid tissues was dependent upon the experience/training that the individual possessed in examining lymphoid tissue and the apparent severity of the specific lesion. In a further study, the accuracy of extended histopathology to detect immunotoxic chemicals was investigated [54]. While, overall, there was good agreement between histopathology and functional tests, the ANTIBODY-forming cell (AFC) assay detected immune suppression in two instances where no changes in pathology were indicated. In contrast, the AFC assay failed to detect oxymetholone as an IMMUNOTOXICANT, although extended histopathology indicated immunological changes. These data suggest that, while not as sensitive as functional tests, extended histopathology may provide a reasonable level of accuracy to identify immunotoxic chemicals. In the NTP protocol, in contrast to the OECD 407 guideline, the high dose was selected so as not to produce overt toxicity, thus limiting the likelihood of producing severe histopathological effects.

Immunotoxicity testing in non-rodent species

Various non-human primates, including *Macaca mulatta* (rhesus macaque), *Macaca nemestrina* (pig-tailed macaque), *Macaca fascicularis* (cynomolgus monkey) and the marmoset have been used in immunotoxicological studies. Virtually all of the immunotoxicology assays that are carried out in the mouse or rat can be and have been adapted for use with the non-human primates [55]. Phenotypic markers and functional assays in three different species of non-human primates were evaluated [56]. Functional assays included NK cell activity, lymphocyte transformation and antigen presentation. The extensive phenotypic marker studies included the evaluation of over 20 markers or combination of markers for each of the three monkey species. Otherwise, strategies and methods applied in studies in humans have been introduced in studies on non-human primates (see Tab. 2).

Other mammalian species have also been used. While dogs are not the species of choice for immunotoxicological studies, they are one of the species predominantly used in toxicological safety assessments. Virtually all of the assays used for assessing immunotoxic potential have been adapted for use in the dog [39]. Among these are assay evaluation of basal Ig levels for IgA, IgG and IgM, allergen-specific serum IgE, mononuclear phagocyte function, NK cell activity, CYTOTOXIC T cell activity, and MITOGEN and cell-mediated immune responses.

Recently, some experience has been gathered in Göttingen-Ellegaard minipigs [57]. Using KLH as a T cell-dependent antigen, the authors characterized the effects of CICLOSPORIN and dexamethasone, revealing a similar response of the minipig IMMUNE SYSTEM to that observed earlier in other non-rodent species. The minipig is a non-clinical species that is receiving growing attention as an additional choice for toxicological testing [58]. Due to the commercial interest in pigs, there is a huge amount of available knowledge on the pig IMMUNE SYSTEM, which is applicable also to the minipig [59].

Procedures for preclinical testing of sensitizing capacity

Structure-activity relationships

The intrinsic capacity of chemicals to exert adverse effects is linked to the structure of the compound. Structure-activity relationships with respect to direct toxicity of compounds to components of the IMMUNE SYSTEM have received little attention. More attention has been given to structure-activity relationships with respect to the induction of ALLERGY. Here, structure-activity relationship models are directed towards a fuller understanding of the relationship between chemical structure and physicochemical properties and skin-sensitizing activity, in order ideally to derive quantitative structure-activity relationships (QSAR), linked perhaps to the development of expert, rule-based systems. In this context, parameters that appear to be of particular importance are protein reactivity and lipophilicity associated with the capacity to pen-

Table 2. Assays recommended for immunotoxicity assessment in humans

1. Complete blood count with differential count
2. Antibody-mediated immunity (one or more of following):
   - · Primary antibody response to protein antigen (e.g., epitope labeled influenza vaccine)
   - · Immunoglobulin concentrations in serum (IgM, IgG, IgA, IgE)
   - · Secondary antibody response to protein antigen (diphtheria, tetanus or polio)
   - · Natural immunity to blood group antigens (e.g., anti-A, anti-B)
3. Phenotypic analysis of lymphocytes by flow cytometry:
   - · Surface analysis of CD3, CD4, CD8, CD20
4. Cellular immunity:
   - · Delayed-type hypersensitivity (DTH) skin testing
   - · Primary DTH reaction to protein (KLH)
   - · Proliferation to recall antigens
5. Autoantibodies and inflammation:
   - · C-reactive protein
   - · Autoantibody titers to nuclei (ANA), DNA, mitochondria and IgE (rheumatoid factor)
   - · IgE to allergens
6. Measure of non-specific immunity:
   - · NK cell enumerations (CD56 or CD60) or cytolytic activity against K562 tumor cell line
   - · Phagocytosis (NBT or chemiluminescence)
7. Clinical chemistry screen

   Proposal for all persons exposed to immunotoxicants

From: [30]

etrate into the viable epidermis [60]. The correlation of the protein reactivity of chemicals with their skin SENSITIZATION potential is well established [61], so that it is now accepted that if a chemical is capable of reacting with a protein, either directly or after appropriate (bio)chemical transformation, it has the potential to be a contact allergen, assuming of course that it can accumulate in the appropriate epidermal compartment. Each of the existing structure-activity relationship (SAR) models proposes structural alerts, i.e., moieties associated with sensitizing activity. In all cases, the structural alerts comprise electrophilic moieties, or moieties that can be metabolized into electrophilic fragments (proelectrophiles).

Testing for skin allergy

Guinea pig models

The guinea pig was for many years the animal of choice for experimental studies of contact SENSITIZATION and several test methods were developed in this species (reviewed in [62]). The best-known and most widely applied are the Buehler test, the guinea pig maximization test, and the guinea pig optimization test, and have formed the basis of hazard assessment for many years. Both the guinea pig maximization test and the Buehler test are now recommended according to an OECD guideline, accepted in 1992.

While these tests differ with respect to procedural details, they are similar in principle. Guinea pigs are exposed to the test material, or to the relevant vehicle. In the Buehler test, both induction and challenge exposures are done topically. The Buehler test is sensitive, but false negatives are frequently observed. The test was improved by occluded application of the test compound. In the guinea pig maximization test, induction involves intradermal and occluded epidermal exposure, and in the optimization test, induction is done by intradermal, challenge by intradermal and occluded epidermal exposure. Adjuvant is employed in the guinea pig maximization test and the optimization test to augment induced immune responses. Challenge-induced inflammatory reactions, measured as a function of erythema and/or edema, are recorded 24 and 48 h later. Classification of sensitizing activity in the guinea pig tests is qualitative and not quantitative. It is based usually upon the percentage of test animals that display macroscopically detectable challenge reactions. Any compound resulting in positive induction in at least 30% of the animals in an adjuvant test is labeled as a sensitizer; in the case of a non-adjuvant test, 15% positivity is sufficient to classify the compound as a sensitizer.

Mouse models

In recent years, increased understanding of the cellular and molecular mechanisms associated with contact ALLERGY has been derived largely from experimental investigations in the mouse [63, 64]. The most important test to be developed in mice is the local lymph node assay (LLNA) [65]. In contrast to the guinea pig assays described above, activity in the LLNA is measured by the primary T cell response in the draining lymph node following TOPICAL application to the mouse ear. Mice are treated daily, for 3 consecutive days, on the dorsum of both ears, with the test material or with an equal volume of vehicle alone. Proliferative activity in draining lymph nodes (measured by the incorporation *in situ* of radiolabeled thymidine) is evaluated 5 days following the initiation of exposure. Currently, chemicals are classified as possessing sensitizing potential if, with one test concentration, a stimulation index of 3 or greater, relative to vehicle-treated controls, is induced. The method has the advantages of short duration and objective measurement of proliferation and minimal animal treatment. In contrast to guinea pig assays, activity is measured as a function of events occurring during the induction, rather than elicitation phase of contact SENSITIZATION.

Risk assessment of sensitizing chemicals requires, besides hazard identification, the assessment of potency. By using dose-response modeling (employment of a regression method that includes determination of the uncertainty margins) in the LLNA test, the potency of sensitizing chemicals can be determined, thus offering a possibility for classification [66]. The LLNA has been developed further to discriminate skin sensitizers from respiratory sensitizers based on the induction of $CD4^+$ T helper subsets (Th1- *versus* Th2-mediated responses) by the analysis of cytokine profiles in draining lymph node cells [67]. Chemicals differ with respect to the types of HYPERSENSITIVITY they induce. Compounds that induce Th1 cells and mediate type IV delayed HYPERSENSITIVITY are generally skin sensitizers. Such responses are associated with the production by draining lymph node cells of IFN-γ. Compounds that induce Th2 cells and mediate type I immediate HYPERSENSITIVITY by the production of IgE and IgG1 are generally respiratory sensitizers, and are associated with the production by draining lymph node cells of high levels of IL-4. However, this is not true in all cases, as skin SENSITIZATION with some low molecular weight compounds such as picrylchloride [68] and toluene diisocyanate (TDI) [69] can induce respiratory HYPERSENSITIVITY with features of type IV HYPERSENSITIVITY in mice. Also, in humans, specific IgE is only detected in a minority of patients suffering from respiratory ALLERGY induced by TDI. In a recent study, it was proposed that, by direct linkage of proliferation and cytokine production, in a dose-response manner, distinction of contact ALLERGENS from respiratory ALLERGENS may be improved [70].

Testing for respiratory allergy

Most of the animal models that are used for studying specific respiratory tract HYPERSENSITIVITY were developed using high molecular weight ALLERGENS, notably proteins. Very few animal models have been developed as predictive tests for hazard identification and

risk assessment in the area of chemically induced respiratory ALLERGY [71]. The majority of these models are based upon ANTIBODY-mediated events. The models differ with regard to the following aspects: the animal species utilized, the route of administration of the agent, the protocol for both induction and elicitation of responses, type of response measured and judgment of significant response.

Guinea pig models

The guinea pig has been used for decades for the study of anaphylactic SHOCK and pulmonary HYPERSENSITIVITY. The guinea pig is similar to humans in that the lung is a major SHOCK organ for anaphylactic responses to ANTIGENS. The guinea pig responds to HISTAMINE and can experience both immediate-onset and late-onset responses. Airway hyperreactivity and eosinophil influx and INFLAMMATION can also be demonstrated in this animal species. Mechanistic studies have been hampered by the lack of reagents needed to identify cells and mediators in respiratory ALLERGY. In addition, the major anaphylactic ANTIBODY is IgG1, whereas it is IgE in humans and other rodent species.

The guinea pig model developed by Karol et al. [72] has proven to be valuable for low molecular weight chemical ALLERGENS. Guinea pigs sensitized by inhalation of free or protein-bound chemical ALLERGENS, such as TDI, will exhibit symptoms of pulmonary HYPERSENSITIVITY following subsequent inhalation challenge. HYPERSENSITIVITY reactions are measured, usually as a function of challenge-induced changes in respiratory rate or alterations in other breathing parameters such as tidal volume. Changes in breathing patterns can also be provoked in dermally sensitized guinea pigs by inhalation challenge with the free chemical. In this approach, it is not necessary to use hapten-protein conjugates.

A tiered approach to hazard assessment in guinea pigs proposed by Sarlo and Clark [73] comprises sequential analyses of physicochemical similarities with known ALLERGENS, the potential to associate covalently with protein, the ability to stimulate ANTIBODY responses and, finally, activity in a model of respiratory HYPERSENSITIVITY in which animals sensitized by subcutaneous injection are challenged by intratracheal instillation.

Mouse models

Models to investigate airway responses to sensitizing compounds have been developed in the mouse and comprise responses mediated by IgE [74] and non-IgE-mediated reactions [68, 69]. These models have not been used so far for predictive purposes.

As discussed earlier, analysis of the cytokine profile in the mouse LLNA may provide information on whether a compound is a respiratory allergen. In the same series of investigations, it was found that TOPICAL administration to mice of chemical respiratory ALLERGENS stimulated a substantial increase in the serum concentration of total IgE, a response not seen with contact ALLERGENS considered to lack the ability to cause SENSITIZATION of the respiratory tract [67]. These observations suggested that it might be possible to identify chemical respiratory sensitizers as a function of induced changes in serum IgE concentration. The advantage of this approach, which forms the basis of the mouse IgE test, is that measurement of a serum protein is required rather than of hapten-specific ANTIBODY.

Investigations suggest that the mouse IgE test may provide a useful method for the prospective identification of chemical respiratory ALLERGENS [71]. It must be emphasized, however, that to date the assay has been evaluated only with a limited number of chemicals and that most of the analyses have been performed in a single laboratory. Difficulties arise from the assumption of IgE mediation of respiratory HYPERSENSITIVITY response in mice. As mentioned earlier, respiratory allergic responses, associated with increased reactivity of airways, may occur by a delayed type IV immune response-inducing compound [68]. For this reason, actual testing of lung functions *in vivo* seems prudent for those chemicals that are known to sensitize, but are unable to produce IgE responses.

Testing for autoimmunity and drug allergy

Drugs may elicit allergic responses to the drug itself (as hapten-CARRIER complex) or induce autoimmune responses. As the distinction between allergic and autoimmune responses induced by HAPTENS may be gradual, depending on multifactorial etiology, it

is difficult if possible at all, to discriminate between allergenic and autoimmunogenic potential of drugs. In addition, clinical outcomes may differ from one drug to another, and from one individual to another. Probably, individual sensibilities and circumstances are decisive here. Predictive models, that mimic the complete development of AUTOIMMUNITY or drug ALLERGY, are not available, and prediction in these cases may depend on a set of well-designed tests.

Induced and genetic models

A range of animal models are available for detecting the potential of compounds to exacerbate induced or genetically predisposed AUTOIMMUNITY [75]. In induced models, a susceptible animal strain is immunized with a mixture of an adjuvant and an autoantigen isolated from the TARGET organ. Examples are adjuvant arthritis, experimental encephalomyelitis and experimental uveitis in the Lewis strain rat.

Examples of spontaneous models of AUTOIMMUNE DISEASE are the BB-rat and the NOD-mouse, which develop autoimmune pancreatitis and subsequently diabetes, and the (NZBxNZW)F_1 mouse or MRL/lpr mouse, which develop pathology that resembles human SYSTEMIC LUPUS ERYTHEMATOSUS. These models are mainly used in the study of the pathogenesis of AUTOIMMUNITY and the preclinical evaluation of immunosuppressive drugs. Very few studies have addressed the potential of these models to assess whether a drug exacerbates autoimmune reactions or disease. An animal model that has been under scrutiny for some time now in this respect is the Brown Norway rat model [76]. In this rat strain, PENICILLAMINE and nevirapine induce various immunological effects (formation of AUTOANTIBODIES) and skin rashes as typical clinical effects. Other suspected drugs (captopril and felmabate) appeared to be ineffective in this model.

All these models may eventually help in determining the underlying mechanisms, but are probably not suitable as stand-alone predictive assays.

Popliteal lymph node assay

Although, currently, no predictive assays have been developed and validated to identify, in the early phases of toxicity testing, the potential of drugs to induce drug ALLERGY or autoimmune responses, it should be noted that available assays to identify contact sensitizers might also be helpful to identify systemic sensitizers. Clinical signs of systemic adverse immune-mediated effects usually become manifest only during advanced clinical development of drugs. The conditions used in routine preclinical toxicological screening are obviously not optimal for the detection of allergic or autoimmunogenic potential of drugs and chemicals (e.g., small animal number, use of outbred animal strains, dynamics of disease development *versus* snapshot determinations, lack of predictive parameters).

AUTOIMMUNITY often results from the association of the compound with normal tissue components, thereby rendering them immunogenic. A variety of chemicals and drugs, in particular the latter, have been found to induce autoimmune-like responses [77]. To detect chemicals that produce this type of reaction, the popliteal lymph node assay (PLNA) in mice is a possible tool. The PLNA [78] is based upon the hyperplasia (increase in weight) of lymph nodes observed in experimental graft-*versus*-host reactions, and has been modified to assess the immunomodulatory potential of drugs. The test substance is injected subcutaneously into one hind footpad, and the contralateral side is either untreated or inoculated with vehicle alone. Comparison of popliteal lymph nodes from both sides allows the effect of the test drug to be measured. Apart from differences in weight, histological evidence of *in vivo* immunostimulatory activity can be discerned. These pseudo-graft-*versus*-host reactions with follicular hyperplasia have been documented in mice for drugs such as diphenylhydantoin, D-PENICILLAMINE and STZ. The assay appears to be appropriate to recognize sensitizing, i.e., allergenic and autoimmunogenic chemicals, as well as non-sensitizing immunostimulating compounds, and has important advantages as it is a simple model based on local reactions that indicate direct immunostimulation with less interference by immunoregulatory mechanisms. So far, many compounds (mainly pharmaceuticals and structural homologues) have been tested in the PLNA, and outcomes (i.e., PLN-index: ratio of weight and cell numbers of PLN of compound-treated over vehicle-injected animals)

appear to correlate well with documented adverse immune effects in humans [79]. Caution must be exercised in the case of autoimmunogenic drugs, such as procainamide, that acts as a prohapten. They are false-negative in the PLNA, and, as such, require co-injection of metabolizing systems (S9 mix of GRANULOCYTES) to become positive.

Results of preliminary interlaboratory validation studies indicate the potential predictive value of the PLNA, or variations of it using other sites of injection, in the mouse [80] and rat [81]. Thus, the direct PLNA seems to be a versatile tool to recognize T cell-activating drugs and chemicals, including autoimmunogenic chemicals, bearing in mind the possible false-negative results with prohaptens. With the adoptive transfer PLNA, sensitized cells are used as probes to detect the formation *in vivo* of immunogenic metabolites of low molecular weight chemicals [79].

A recent modification of the PLNA uses bystander ANTIGENS to report the nature and type of immune response that is elicited by a given pharmaceutical [82]. In this assay, so-called reporter ANTIGENS (RAg), either TNP-FICOLL or TNP-ovalbumin (TNP-OVA), are injected together with the compound of interest and the IgG response to the RAg is measured. Here, it is important that, unlike TNP-OVA, TNP-FICOLL cannot directly induce specific T cell activation. The IgG response to TNP-FICOLL, however, is susceptible to neo-antigen-specific T cell help, implying that an IgG response to TNP-FICOLL indicates that a co-injected compound has immunosensitizing potential. If a compound increases an IgG response to TNP-OVA and not to TNP-FICOLL, it can be concluded that the compound has the capacity to act merely as an adjuvant to an immune response. Thus, dependent on the type of RAg, the IgG measured in this so-called RAg-PLNA indicates whether compound-induced immunostimulation involves immunosensitization or proinflammatory adjuvant activity. The RAg approach can also be applied in animal tests that use oral exposures to drugs [83].

It is important to note that the PLNA in any of its forms is essentially a hazard identification test that can indicate whether a compound has the potency to induce allergic or autoimmune phenomena in man.

Procedures for immunotoxicity testing in humans

Epidemiology design

It is obvious that many of the compounds causing direct immunotoxicity have been identified in rodent studies, as the database in humans is less complete and often inconclusive. The most common design used in immunotoxicity research in humans is the cross-sectional study, in which exposure parameters and effect parameters are assessed at the same time point [84]. The immune function of "exposed" subjects is compared with the immune function of "non-exposed" subjects by the measurement of various immunological parameters. For this reason, proper definition of exposure criteria in the exposed group is necessary. This group should include subjects at the upper end of exposure. Where possible, the study should incorporate individual estimates of exposure or actual measurements of the compound. In the broadest sense, biomarkers are measurements on biological specimens that will elucidate the relationship between environmental exposure and human diseases, so that exposure and diseases can be prevented. In clinical medicine, biomarkers are valued as surrogates for the presence or absence of diseases or the course of the disease during therapeutic intervention. As such indicators are available for exposure, effect or susceptibility [85].

Markers of exposure

A biological marker of exposure is the presence of a xenobiotic compound or its metabolite or the product of an interaction between the compound and some TARGET cell or biomolecule. The most common markers of exposure are the concentration of the compound in urine, blood or TARGET organ or tissue. Immune-specific biomarkers of exposure are ANTIBODIES or positive skin tests to the particular compound.

Markers of effect

A biomarker of effect is a measurable cellular or biochemical alteration within an organism that,

depending on magnitude, can be recognized as an established or potential health impairment or disease. These range from markers of slight structural or functional changes to markers that are indicators of a subclinical stage of a disease or the manifestation of the disease itself. Functional changes in cells of the IMMUNE SYSTEM by an immunotoxic chemical may be the first step in the process towards disease. For instance, longitudinal studies on asymptomatic individuals with low NK activity showed that they had an increased risk for upper respiratory infection and morbidity [86]. IMMUNOSUPPRESSION may lead to more subtle changes in resistance to infections, such as influenza or common cold, rather than OPPORTUNISTIC INFECTION. Data in experimental animals also indicate that small changes in immune function could increase the likelihood of disease [52].

Markers of susceptibility

Markers of susceptibility, also called effect modifiers, can act at any point along the exposure-disease continuum. Important sources of variability are genetic, endocrine, age-related and environmental factors. Over the last two decades it has become clear that many immunological disorders are linked to alleles of the MHC. The products of MHC alleles in humans [human lymphocyte ANTIGENS (HLA)] have aroused interest at a clinical level as potential biomarkers of disease susceptibility. In some instances, there is a remarkable increase in relative risk of disease in individuals possessing particular alleles. Similar associations have been described in drug-induced immunological disorders. However, it should be noted that other genetic factors as well as environmental factors are also of importance. Stress of various types can also affect the IMMUNE SYSTEM and influence the susceptibility to and recovery from infectious, autoimmune and neoplastic diseases. Age-related variability is shown by the developing fetus, which is more susceptible to immunotoxic effects than is the adult.

Assays for assessment of immune status

There is a plethora of tests developed to assess immunity in humans [87, 88], as described in laboratory manuals [89–91]. Many of these tests are nowadays commercially available as kits. A systematic approach to the evaluation of immune function, which is based on simple screening procedures followed by appropriate specialized tests of immune function, usually permits the definition of the immune alteration. This should include evaluation of the B cell system, of the T cell system and of nonspecific resistance (POLYMORPHONUCLEAR LEUKOCYTES, MONOCYTES and MACROPHAGES, NK cells, the COMPLEMENT SYSTEM).

Testing schemes for evaluation of individuals exposed to immunotoxicants are proposed, among others, by the Subcommittee on Immunotoxicology of the U.S. National Research Council [85] and by a task group of the World Health Organization (WHO) [39]. The panel proposed by the WHO is listed in Table 2, and is composed of assays that cover all major aspects of the IMMUNE SYSTEM. Included are functional assays to test for humoral immunity, i.e., specific ANTIBODIES to tetanus or diphtheria (for which vaccination programs exist), and for cellular immunity using recall ANTIGENS. It should be mentioned that these tests were all developed for diagnostic purposes, but in the context of immunotoxicity testing in humans, they are to be used in an epidemiological setting. This means that distinctions found in parameters between an exposed group and a control group may have a different biological significance than an altered value in an individual. Whereas a decrease in a single immune parameter in an individual may not indicate increased susceptibility to disease, a subtle alteration in an immune biomarker in a population may indicate immunotoxicity.

Establishing immune changes in humans is considerably more complex than in animals, considering that non-invasive tests are limited, exposure levels to the agent (i.e., dose) are difficult to establish and responses in the population are extremely HETEROGENEOUS. Also, the normal population exhibits a wide range of immunological responses with no apparent health impact. In addition to this underlying population variability, certain host characteristics or common exposures may be associated with significant, predictable alterations in immunological parameters. If not recognized and effectively addressed in the study design or statistical analysis, these confounding factors may severely alter the results

of population studies. Examples of factors associated with measurable alterations in immunological parameters include age, race, gender, pregnancy, acute stress and the behavioral ability to cope with stress, coexistent diseases or infections, nutritional status, lifestyle, tobacco smoking and some medications. Besides these variables, periodical (ranging from daily to seasonal) influences also exist. Some of these effects are relatively minor; other differences may be sufficiently large to exceed the expected effect from a low level of immunotoxic exposure. They are therefore of primary concern in large epidemiological studies. For a review on the influence of endogenous and environmental factors on vaccination responses, see [92]. The importance of genetic factors for the response to a vaccine is shown by the role of cytokine polymorphisms in the susceptibility of humans to ultraviolet B-induced modulation of immune responses after hepatitis B vaccination [93].

Predictive testing for allergy in humans

There are a variety of skin test procedures for the diagnosis of several types of allergic reactions, dealt with above. Basically, predictive tests in humans for skin ALLERGY are similar to diagnostic tests, but the aims are different (see chapter B1). For diagnostic tests, the aim is to determine SENSITIZATION to chemicals to which there has been a prior exposure, whereas SENSITIZATION as a result of the procedure should be avoided. For predictive testing in humans, the aim is to show sensitizing capacity in individuals who have not been exposed previously to the compound.

For obvious reasons, predictive testing for respiratory SENSITIZATION is not done in humans. Occasionally, case reports may serve as adequate hazard identifications, but not as a risk estimate, because data on route and extent of exposure, and on the "population at risk" are usually missing. In the absence of case reports, it cannot be concluded that no potential for SENSITIZATION exists.

Immunotoxicity regulations

Regulatory guidance

There is great variation in the approaches adopted by regulatory agencies throughout the world to the control of human pharmaceuticals. Leading agencies involved in the regulation of pharmaceuticals for human use are the U.S. Food and Drug Administration (FDA), the Committee on Human Medicinal Products (CHMP) of the European Medicines Agency (EMA) in the European Union (EU), and the Ministry of Health, Labor and Wellfare (MHLW), with the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan. The requirements the industry has to meet, in order to gain marketing approval for its products, have been laid down in official guidelines. These guidelines inform the industry about the data needed to demonstrate to the authorities the pharmaceutical quality of new pharmaceuticals to be marketed, as well as their benefit and safety for the patient. The guidance given has a major impact on the development programs adopted by the industry.

The regulations administered by government agencies are greatly influenced by the history, culture and legislations of the countries concerned. This still accounts for many national differences [94, 95]. However, worldwide harmonization of regulatory requirements is ongoing. Since the 1990s, the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has proved to be a success. In this international forum, government regulators and industry representatives of the three major regions of the world pharmaceutical market participated (i.e., United States, the EU, and Japan). A number of harmonized guidelines have been developed by the ICH. Most of these have already been adopted officially by the regulatory authorities mentioned. Existing national guidelines thus have been or will be replaced by new ones based on the ICH consensus.

Regulatory aspects of laboratory animal immunotoxicology

To identify potential TARGET organs of toxicity in humans, the industry must screen the toxicity of pharmaceuticals in laboratory animals. Among targets such as liver and kidney, adverse effects on the IMMUNE SYSTEM need to be assessed. As a rule, the regulatory authorities do not dictate how specific tests have to be conducted. The detailed technical requirements defined by the OECD may or may not be followed. This approach allows deviations from routine protocol toxicity testing whenever justified. The study protocols may be adjusted in such a way that they provide the most relevant information, depending on the nature and therapeutic indication of the pharmaceutical to be tested. Guidelines on immunotoxicology were introduced a decade ago (2000–2001) in the EU and the United States (a draft status only in Japan), which focus particularly on immunotoxicological issues regarding pharmaceuticals. In this regard, it is necessary to differentiate between the various aspects of immunotoxicity, i.e., IMMUNOSUPPRESSION, ALLERGY and AUTOIMMUNITY. Within the framework of ICH, a harmonized guideline has been written based on a CAUSE-FOR-CONCERN APPROACH (ICH, 2006) (see below).

Unintended immunosuppression

Current predictive immunotoxicity testing is mainly done in the context of general toxicity, according to OECD guideline 407. Tests on the IMMUNE SYSTEM comprise hematology, including differential cell counting, and histopathology of lymphoid tissues. Within the OECD, a debate over functional testing, i.e., measurement of ANTIBODY responses to SRBC, is in progress, in addition to discussion regarding the inclusion of measurement of NK activity and inclusion of FACS analysis of lymphocyte subpopulations.

The guidelines from all regions followed similar lines for immunotoxicity testing as those drawn up by the OECD. The major difference between the EU and the guidance in the other regions is that the CHMP had included functional testing in routine screening, mentioning explicitly the primary ANTIBODY response to a T cell-dependent antigen. Measuring NK activity, together with FACS analysis of lymphocyte subset populations, might be used as an alternative approach. Such testing has been instrumental in identifying compounds that induce unintended immune suppression. The FDA document advocates a CAUSE-FOR-CONCERN APPROACH on the need for functional assays. A cause-for-concern is generally an adverse effect found in the toxicity endpoints, but might also be the intended use or the pharmacological activity of compounds (e.g., anti-HIV drugs, or anti-inflammatory activity).

Under the auspices of the ICH, a process was started in November 2003 to harmonize the approach to screening human pharmaceuticals with regard to unintended IMMUNOSUPPRESSION. The industry provided a small database of pharmaceutical compounds being tested with respect to immune function, which has been gathered in two steps, and analyzed [96]. Of 64 compounds, 54 were included in a formal evaluation. The main question was: would additional immunotoxicity testing be more sensitive than the immunological endpoints of standard toxicity testing? Eventually, only 6 of 45 compounds could be called immunotoxic on the basis of additional immunotoxicity testing, without evidence in standard toxicity studies. It was decided that this number was too low to justify a standard requirement for functional testing, as was commonly requested by the EU at that time. Therefore, a CAUSE-FOR-CONCERN APPROACH was adopted. Factors that might prompt additional immunotoxicity studies that were agreed upon were: (1) findings from standard toxicity studies (see Box 1), (2) the pharmacological properties of the drug, (3) the intended patient population, (4) structural similarities to known immunomodulators, (5) the disposition of the drug, and (6) clinical information. (ICH, 2006) [97]. A list of additional studies mentioned in the ICH guidance document is given in Table 3. A weight of evidence review is needed to justify the absence of additional immunotoxicity studies. If the outcome of the assessment of the drug-induced immunotoxicity suggests a risk of immunotoxicity, it should be weighed whether this risk is considered acceptable and/or should be addressed clinically in a so-called Risk Management Plan. In the latter case, no further animals studies are needed.

Box 1. ICH S8 Note for Guidance on Immunotoxicity studies for Human Pharmaceuticals

Objectives of the Guideline
The objectives of this guideline are to provide (1) recommendations on nonclinical testing approaches to identify compounds that have the potential to be immunotoxic, and (2) guidance on a weight-of-evidence decision making approach for immunotoxicity testing. Immunotoxicity is, for the purpose of this guideline, defined as unintended immunosuppression or enhancement. Drug-induced hypersensitivity and autoimmunity are excluded.

Overview
The general principles that apply to this guideline are:
i) All new human pharmaceuticals should be evaluated for the potential to produce immunotoxicity.
ii) Methods include standard toxicity studies and additional immunotoxicity studies conducted as appropriate. Whether additional immunotoxicity studies are appropriate should be determined by a weight of evidence review of the following factor(s) that should be considered:
- Standard toxicity studies
- Pharmacological properties
- Intended patient population
- Structural similarity
- Disposition of the drug
- Signs observed in clinical trials or clinical use

Regarding standard toxicity studies
Changes in these parameters could reflect immunosuppression or enhanced activation of the immune system. Immunosuppression is usually reflected by reduced values of immune parameters, whereas immunoenhancement is usually reflected by increased values. However, these relationships are not absolute and can be inverted in some cases. Similar to the assessment of risk with toxicities in other organ systems, the assessment of immunotoxicity should include the following:
- Statistical and biological significance of the changes
- Severity of the effects
- Dose/exposure relationship
- Safety factor above the expected clinical dose
- Treatment duration
- Number of species and endpoints affected
- Changes that may occur secondarily to other factors (e.g., stress)
- Possible cellular targets and/or mechanism of action
- Doses which produce these changes in relation to doses which produce other toxicities
- Reversibility of effect(s).

Regarding Pharmacological Properties
If the pharmacological properties of a test compound indicate it has the potential to affect immune function (e.g., anti-inflammatory drugs), additional immunotoxicity testing should be considered. Information obtained from the nonclinical pharmacology studies on the ability of the compound to affect the immune system could be used in a weight of evidence approach to decide if additional immunotoxicity studies are needed.

Weight of evidence review
A weight of evidence review should be performed on information from all the factors outlined above to determine whether a cause for concern exists. A finding of sufficient magnitude in a single area should trigger additional immunotoxicity studies. Findings from two or more factors, each one of which would not be sufficient on its own, could trigger additional studies. If additional immunotoxicity studies are not performed, the sponsor should provide justification.

Table 3. List of additional studies mentioned in the ICH S8 document

Additional immunotoxicity studies: (AIS)
T cell dependent antibody response (TDAR)
Immunophenotyping
Natural killer cell activity assays
Host resistance models
Macrophage/neutrophil function
Assay to measure cell-mediated immunity

Sensitizing capacity

Regulatory HYPERSENSITIVITY testing generally focuses on locally applied compounds. Guidance documents focus predominantly on Type IV HYPERSENSITIVITY. The EMA/CHMP Note for Guidance on Local TOLERANCE testing refers to the OECD guidelines with regard to test methods. The OECD test 406 guideline on skin SENSITIZATION covers the guinea pig maximization test and the Buehler test; OECD guideline 429 covers the LLNA. The FDA and CHMP regard the LLNA as a suitable stand-alone method for detecting HYPERSENSITIVITY potential. The guidance document of the MHLW requests that all dermatological preparations should be tested in at least one skin SENSITIZATION study. The following tests are regarded as acceptable by the MHLW; Adjuvant and Patch test, Buehler test, Draize test, Freud's complete adjuvant test, maximization test, open cutaneous test, optimization test and the split adjuvant test. The most commonly used test in Japan is the Maximization test, followed by the Adjuvant Patch test or the Buehler test. Table 4 provides an overview of the regional requirements.

Drugs intended for inhalation should be tested for their potential to induced Type I HYPERSENSITIVITY reaction according to the FDA. The Japanese and the European authorities have not issued a non-clinical requirement for inhalation ALLERGY testing.

Table 4. Test requirements for topically applied drugs

Assays	EMA	FDA	MHLW
GPMT	All[A]	All[A]	All[A]
BA	All[A]	All[A]	All[A]
LLNA	All[A]	All[A]	All[A]
MEST	All	All	All[A]
Adjuvant and patch test	-	-	All[A]
Draize test	-	-	All[A]
FCA	-	-	All[A]
Open epicutaneous test	-	-	All[A]
Optimization test	-	-	All[A]
Spilt adjuvant test	-	-	All[A]
MIGET	-	Inhalation drugs	-

All drugs: All topically applied drugs
All drugs[A]: test regarded as stand-alone assay
GPMT, guinea pig maximization test; BA, Buehler assay; LLNA, local lymph node assay; MEST, mouse ear swelling test; FCA, Freund's complete adjuvant test; MIGET, multiple inert gas elimination technique

Autoimmunity

Because of the lack of suitable and validated animal models, the potential risk for inducing AUTOIMMUNITY cannot be predicted. As regulatory guidance follows scientific developments, such guidance for AUTOIMMUNITY is not available. The FDA guidance document touches the issue of AUTOIMMUNITY, mentioning the PLNA as a proposed test for AUTOIMMUNITY. It recognizes, however, that an extensive evaluation to support its use is currently lacking. Biomarkers of T cell activation or markers of Th2 cell induction and autoantibody induction in experimental animals may be helpful, but their predictive value for determining the potential to induce AUTOIMMUNE DISEASE in humans has not been ascertained. Neither the EMA nor the MHLW has released any guidance on AUTOIMMUNITY testing.

Immunotoxicology of biotechnological products

In 1997, the ICH issued a guideline on "Preclinical Safety Assessment of Biotechnology-Derived Pharmaceuticals", which has been adopted and implemented by all three regions (for the EMA link see below). This document contains guidance on the assessment of immunogenicity as many products of this type are immunogenic in animals. ANTIBODY responses should be characterized, and their appearance should be correlated with any pharmacological and/or toxicological changes. The finding of ANTIBODIES against the product should, however, not be the sole criterion for the early termination of an animal study. Only in the case of neutralization of the response in the majority of the animals should the study be stopped. It should be kept in mind that ANTIBODY formation in animals against a human protein is generally not predictive of a potential for ANTIBODY formation in humans. In the updating of the Guideline that is currently in progress during the preparation of this chapter, it has been proposed not to characterize the anti-drug-ANTIBODY response routinely, but only if there is evidence for any change in the pharmacological response of the product. This reflects the growing insight that immunogenicity of human proteins in animals, as such, is not predictive for the human situation, and testing for anti-drug-ANTIBODIES is only needed to understand the outcome of the toxicity studies performed.

Little specific information is present on general immunotoxicity. Inflammatory responses might be indicative of a stimulatory immune response, but may also be the result of toxic changes. Routine tiered testing approaches are not recommended for biotechnology-derived pharmaceuticals, as it is supposed that, on the one hand, the character of most of RECOMBINANT proteins is well-known and not directed to the IMMUNE SYSTEM. On the other hand, the effects on the IMMUNE SYSTEM of immunosuppressive MONOCLONAL ANTIBODIES will be tested anyway by characterizing their pharmacodynamics.

The ICH S6 document is the first one to mention developmental immunotoxicity, but again the guidance is very general, indicating that the study design of the toxicity studies may be modified, e.g., in the case of MONOCLONAL ANTIBODIES with prolonged immunological effects. The more recent FDA guidance suggests the incorporation of immunotoxicological determinations in the ICH Stages C–F reproductive toxicology study if a drug is expected to be used in pregnant women. Guidance documents from the other areas do not specifically address developmental toxicity.

The lack of detailed instructions is due to the fact that all three regions concerned have adopted a flexible, case-by-case, science-based approach to animal toxicity studies in this rapidly evolving scientific area. Consequently, the three regions involved in ICH are reluctant to formulate in-depth requirements that may turn out to be too strict. Such guidance might be valid for some biotechnology-derived products, but not for others.

Antigenicity

The Japanese Guidelines for Nonclinical Studies of Drugs require antigenicity studies to be conducted on a case-by-case basis for conventional drugs. There is a lot of resistance to this request of the MHLW. Just recently, a representative of the Japanese government indicated that they believe antigenicity data for con-

ventional compounds are not predictive for any risks of these compounds, which promises to be a first step in deleting this issue from the guidance.

New developments

Recently, the National Institute of Environmental Health Sciences NIEHS and the National Institute of Occupational Safety and Health (NIOSH) have started a discussion on a potential consensus document on the most appropriate experimental approaches and assays available to assess developmental immunotoxicity [98]. This initiative is also supported by the International Life Sciences Institute (ILSI) in Washington, which organized a conference on this topic in 2003. Examples of pharmaceuticals as developmental immunotoxicants in rodents are acyclovir, CICLOSPORIN, cyclophosphamide, corticosteroids, benzodiazepines, AZATHIOPRINE and TACROLIMUS. As mentioned above, the FDA suggests incorporation of immunotoxicological determinations in reproductive toxicology studies.

In the future, transgenic animals or adoptive transfer models might contribute to our insight into the immunotoxic mechanisms of compounds *in vivo*. Further contributions to the screening for immunotoxicity can be expected from toxicogenomics (MICROARRAY technology) and PROTEOMICS.

Importantly, as with other toxicological disciplines results of tests should be translatable to the organism of interest, taking into account the relevance of test animals or *in vitro* methods [99]. Translational aspects of hazard and risk evaluation, although not new, will receive more attention in coming years, in particular in the field of immunotoxicology.

Conclusions

Preclinical testing of pharmaceuticals in laboratory animals requires different approaches to detect direct immunotoxicity, resulting in unwanted IMMUNOSUPPRESSION or immunostimulation, or to detect drug-induced HYPERSENSITIVITY and AUTOIMMUNITY. Tiered immunotoxicity-testing procedures have been developed and validated in the rat and mouse, and are being used successfully to detect drug-induced direct immunotoxicity.

Drug-induced HYPERSENSITIVITY and autoimmune reactions are of great clinical concern. For contact ALLERGY, routine contact SENSITIZATION testing in guinea pigs has been extended by the LLNA in the mouse as a stand-alone method for detecting HYPERSENSITIVITY potential. An important issue in contact ALLERGY is the development of quantitative measurements of the potency of ALLERGENS. No validated models are yet available to investigate the ability of drugs to induce respiratory SENSITIZATION. The LLNA in mice or skin SENSITIZATION testing in guinea pigs should be recommended as a first screen.

Animal models are currently available to detect the potential of compounds to exacerbate induced or genetically predisposed AUTOIMMUNITY, but are seldom used in immunotoxicity studies. Models to investigate the ability of chemicals to induce AUTOIMMUNITY, as a result of an immune response to self-proteins modified by the chemical, are virtually limited to the PLNA. As human data show that chemical agents, in particular drugs, can cause AUTOIMMUNE DISEASES, new models should be developed.

In conclusion, immunotoxicology is a rapidly evolving field in the regulation of pharmaceuticals. This is reflected in the rapid harmonization of the regional approaches on immunotoxicity testing under the auspices of ICH. The standard use of a large immunotoxicological test battery is not recommended. Instead, a flexible approach on a case-by-case basis is accepted, taking into account that some classes of drugs and some indications may be a greater cause of concern than others.

Important websites

Guidelines on hypersensitivity testing:
http://oecdpublications.gfi-nb.com/cgi-bin/OECDBookShop.storefront/
ILSI-HESI information on immunotoxicology:
http://www.hesiglobal.org/i4a/pages/index.cfm?pageid=3471

FDA guidelines:
http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080495.htm
http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079239.pdf
EMEA guidelines:
http://www.ema.europa.eu/pdfs/human/swp/214500en.pdf
http://www.ema.europa.eu/pdfs/human/ich/030295en.pdf

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Appendices

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Appendix C1. Selected examples of vaccines registered in the USA

Generic name	Trade name, manufacturer	Antigens and antigen dose	Main excipients	How supplied	Recommended dosage and administration
Anthrax vaccine adsorbed	Biothrax, Emergent Biodefence Corporation	Proteins from avirulent *Bacillus anthracis* strain	Aluminium hydroxide, benzethonium chloride, formaldehyde	Liquid, multidose vial	0.5 mL, 2× s.c., given 4 weeks apart followed by 3× s.c., given at 6, 12, and 18 months Risk groups only, individuals 18–65 years of age
Diphtheria and tetanus toxoids adsorbed, for paediatric use	(Generic), Sanofi Pasteur	DT (6.7 Lf), TT (5 Lf)	Aluminium potassium s ulphate, NaCl, phosphate buffer	Liquid; single-dose vial	0.5 mL, 2× i.m. 4-8 weeks apart followed by 1× 6–12 months after first injection For children 6 weeks – 7 years of age
DTaP, adsorbed	Infanrix, Glaxo-SmithKline Biologicals	DT (30 IU), TT (40 IU), 3 pertussis antigens (25 µg PT, 25 µg FHA, 8 µg pertactin)	Aluminium salts, NaCl, 2-phenoxyethanol	Liquid; single-dose vial	0.5 mL, 3× deep i.m., at least 4 weeks between doses For children 2 months – 6 years of age
DTaP-IPV-HepB, adsorbed	Pediarix, Glaxo-SmithKline Biologicals	DT (25 Lf), TT (10 Lf), 3 pertussis antigens (25 µg PT, 25 µg FHA, 8 µg pertactin), HBsAg (10 µg), poliovirus types 1,2,3 (40, 8 and 32 DU, respectively)	Aluminium salts, NaCl, 2-phenoxyethanol	Liquid; single-dose vial or syringe	0.5 mL, 3× i.m., at 6–8-week intervals Customary age for first dose: 2 months of age
HiB-HepB vaccine	COMVAX, Merck&Co	Haemophilus b conjugate (7.5 µg PRP, conjugated to meningococcal protein) and HBsAg (5 µg)	Aluminium hydroxy-phosphate sulphate, sodium borate, NaCl	Liquid, single-dose vial	0.5 mL, 3× i.m., ideally at 2, 4, and 12–15 months of age
HiB vaccine	ActHIB, Sanofi Pasteur	Haemophilus b conjugate (10 µg PRP, conjugated to 24 µg TT)	Sucrose, NaCl	Lyophilised, single-dose vial Diluent: 0.4% NaCl Reconstitution with DTP possible	0.5 mL, 4× i.m., ideally at 2, 4, 6, and 15–18 months of age

Appendix C1. Selected examples of vaccines registered in the USA (continued)

Generic name	Trade name, manufacturer	Antigens and antigen dose	Main excipients	How supplied	Recommended dosage and administration
HepA-HepB vaccine	TWINRIX, GlaxoSmith-Kline Biologicals	Inactivated HepA virus (720 ELISA units), HBsAg (20 μg)	Aluminium phosphate and hydroxide, amino acids, 2-phenoxyethanol, NaCl, phosphate buffer, Tween 80	Liquid, single-dose vial or syringe	1.0 mL, 3× i.m. on a 0-, 1-, and 6-month schedule Risk groups only, individuals from 18 years of age
HepA vaccine	VAQTA, Merck&Co	Inactivated HepA virus (25 units, paediatric; 50 units, adults)	Aluminium hydroxyphosphate sulphate, sodium borate, NaCl	Liquid, single-dose vial	2–18 years of age: 0.5 mL, 2× i.m. on month 0 and 6–18 As of 19 years of age: 1.0 mL, 2× i.m. on month 0 and 6–18
HepB vaccine	Engerix-B, GlaxoSmith-Kline Biologicals	HBsAg (10 μg)	Aluminium hydroxide, NaCl, phosphate buffer	Liquid, single-dose vial or syringe	0.5 mL, 3× i.m. on a 0-, 1-, and 6-month schedule
HPV vaccine	Cervarix, GlaxoSmith-Kline Biologicals	Virus-like particles of L1 protein of HPV strains 16 and 18	Aluminium hydroxide, Monophosphoryl lipid A, NaCl	Liquid, single-dose vial or syringe	0.5 mL, 3× i.m. on a 0-, 1- and 6-month schedule for females between 10 and 25 years of age
Influenza virus vaccine, trivalent, types A and B	Fluzone, Sanofi Pasteur	Haemagglutinin (3× 15 μg) from 3 influenza virus strains	NaCl, phosphate buffer, thiomersal, gelatin	Liquid, single-dose vial or syringe	0.5 mL, 2× i.m., at least 1 month apart Not approved for infants <6 months of age
Japanese encephalitis virus vaccine inactivated	JE-Vax	Inactivated virus	Sucrose, gelatin, thiomersal	Lyophilised, single-dose or multi-dose vial Diluent: water for injection	0.5 mL (1–3 years of age) or 1.0 mL (>3 years of age), 3× s.c. on day 0, 7, and 30; booster after 2 years
Measles, mumps, and Rubella virus vaccine, live	M-M-R II, Merck & Co	Live attenuated measles, mumps and rubella viruses	Sorbitol, sodium phosphate, NaCl, hydrolysed gelatin, human albumin	Lyophilised, single-dose vial Diluent: water for injection	0.5 mL, 2× s.c. at 12–15 months of age and 4–6 years of age

Meningococcal polysaccharide vaccine, groups A, C, Y and W-135 combined	Menomune-A/C/Y/W-135, Sanofi Pasteur	Meningococcal polysaccharides groups A, C, Y, and W-135 (50 µg each)	Lactose, NaCl	Lyophilised, single-dose or multi-dose vial Diluent: water for injection (with thiomersal for multi-dose vial)	0.5 mL, s.c., for both adults and children, revaccination may be considered after 3–5 years
Pneumococcal vaccine, conjugate	Prevnar 13, Wyeth Pharmaceuticals	13 pneumococcal polysaccharides (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 18C, 19A, 19F and 23F (2.2 µg each, 4.4 µg of 6B) individually conjugated to diphtheria CRM197	Aluminium phosphate	Liquid, single-dose vial	0.5 mL, 3× i.m. separated by 2-month intervals for infants <6 months of age, followed by second dose in 2nd year of age
Rabies vaccine	RabAvert, Novartis Vaccines & Diagnostics	Inactivated virus (at least 2.5 IU of rabies antigen)	Buffered polygeline, potassium glutamate	Lyophilised, single-dose vial Diluent: water for injection	1.0 mL, 3× i.m. on days 0, 7, and 21 or 28
Smallpox vaccine	Dryvax, Wyeth Lederle	Attenuated live vaccinia virus (ca. 10^8 infectious viruses/mL)	Glycerin, phenol	Lyophilised, multi-dose vial Diluent: 50% glycerin, 0.25% phenol in water for injection	0.25 mL, into superficial skin layers using a bifurcated needle Risk groups only; recommendations in response to bioterrorism: http:/www.cdc.gov
Typhoid vaccine Live Oral Ty21a	Vivotif, Berna Biotech	Live attenuated *Salmonella typhi* Ty21a (2×10^9–6×10^9 colony-forming units)	Sucrose, lactose, ascorbic acid, amino acids, magnesium stearate	Enteric-coated capsule	Capsule, 4× orally, 1 h before a meal with a cold or luke-warm drink, on alternate days Risk groups only, >6 years of age
Typhoid Vi Polysaccharide vaccine	TYPHIM Vi, Sanofi Pasteur	Polysaccharide (25 µg) from *Salmonella typhi* strain Ty2	NaCl, phosphate buffer	Liquid, single-dose syringe	0.5 mL, 1× i.m., reimmunisation every 2 years Risk groups only, >2 years of age

Appendix C1. Selected examples of vaccines registered in the USA (continued)

Generic name	Trade name, manufacturer	Antigens and antigen dose	Main excipients	How supplied	Recommended dosage and administration
Rotavirus vaccine	Rotateq, Merck&Co	Five live attenuated reassorted rotavirus strains	Sucrose	Liquid, single-dose tube	2 mL, 3× oral for children 6–32 weeks of age (first dose between 6 and 12 weeks of age)
Varicella vaccine	Varivax, Merck&Co	Live attenuated varicella virus (>1350 plaque-forming unites)	Sucrose, hydrolysed gelatin, NaCl, sodium glutamate, phosphate buffer, KCl	Lyophilised, single-dose vial Diluent: water for injection	0.5 mL, 1–2× s.c. Not indicated for children <1 year of age
Yellow Fever vaccine	YF-Vax, Sanofi Pasteur	Live attenuated yellow fever virus (>$5.5*10^4$ plaque-forming units)	Sorbitol, gelatin, NaCl	Lyophilised, single-dose vial Diluent: isotonic NaCl	0.5 mL, 1× s.c. Risk groups only, >9 months of age

CRM_{197}, nontoxic variant of diphtheria toxin; DT, diphtheria toxoid; DTP, diphtheria-tetanus-pertussis combination vaccine; DU, D-antigen units; FHA, filamentous haemagglutinin; HBsAg, recombinant hepatitis B surface antigen; HepA, hepatitis A; HepB, hepatitis B; HiB, Haemophilus influenzae *type B; HPV, human papillomavirus; IU, international units; Lf, limits of flocculation (flocculation units); PRP, polyribosylribitol phosphate; PT, detoxified pertussis toxin; TT, tetanus toxoid*

Appendix C3. Marketed anti-allergic products

Chemical name	Trade name	Formulation	Usual dose	Pharmacokinetics	Indications	Contra-indications
Cromones						
Nedocromil	Tilade	Dose aerosol	2 mg/inhalation 4× 4 mg/day	Complete resorption from lungs. Not metabolised. Eliminated unchanged, 70% urine, 30% faeces. $T_{1/2el}$ = 1–2 h	Prevention of allergic or exercise induced asthma	
Nedocromil	Tilavist	Eye drops	20 mg/mL 1 drop 2 times/ day/per eye	See above	Prevention of allergic conjunctivitis	
Disodium cromoglycate	Lomudal	Dose aerosol Inhalation powder Inhalation liquid	5 mg/inhalation 4× 10 mg/day 20 mg/capsule 4× 20 mg/day 20 mg/2 mL4× 20 mg/day	Complete resorption from lungs. Not metabolised. Eliminated unchanged, 50% urine, 50% gal. $T_{1/2el}$ = 1.5–2 h.	Prevention of allergic or exercise induced asthma	
Disodium cromoglycate	Lomusol	Nose drops	20 mg/mL 4–6 drops/day	See above	Prevention of allergic rhinitis	Preservative sensitivity
Disodium cromoglycate	Lomusol Otrivin Prevalin Vividrin Allergocrom	Nose spray	20 mg/ml 4–6 sprays/day	See above	Prevention of allergic rhinitis	Preservative sensitivity
Disodium cromoglycate	Opticrom Prevalin Vividrin Allergocrom	Eye drops	20 mg/mL 1–2 drops, 4–6 times/day	See above	Allergic conjunctivitis	Preservative sensitivity
Disodium cromoglycate	Nalcrom	Drink	200 mg 4 times/day	See above	Food allergy	

Appendix C3. Marketed anti-allergic products (continued)

Chemical name	Trade name	Formulation	Usual dose	Pharmacokinetics	Indications	Contra-indications
H_1-receptor antagonists						
Clemastine	Tavegil	Injection liquid Tablet	1 mg/mL, 2 mL 1 mg	Well absorbed. T_{max} 5–7 h, duration 12 h. Metabolised in liver. $T_{1/2el}$ = 20–24 h.	Treatment of anaphylactic shock	Children under 1 year of age
Dimethindene	Fenistil	Capsule 1 mg	1–2 mg three times/day	Well absorbed. Metabolised in liver. $T_{1/2el}$ = 20–24 h.	Allergic conditions	Prostate hyperplasia, urine retention, glaucoma, new born babies
Dexchloro-feniramine	Polaramine	Tablet 2 mg Syrup 0.4 mg/mL	Max. 12 mg/day	Well absorbed. T_{max} 2 h. Metabolised in liver. $T_{1/2el}$ = 6 h.	Allergic conditions	Prostate hyperplasia, pylorus stenosis
Emedastine	Emadine	Eye drops 0.5 mg/mL	1 drop twice a day	Well absorbed. T_{max} 1–2 h. Metabolised in liver. $T_{1/2el}$ = 10 h.	Rhinitis/ urticaria	Preservative sensitivity
Hydroxyzine	Atarax Navicalm	Tablet, 10, 25, 100 mg Syrup 2 mg/mL	50–100 mg/day	Well absorbed. T_{max} 2 h, duration 4–6 h. Metabolised in liver. $T_{1/2el}$ = 2 0h.	Pruritis/ urticaria	
Mebhydroline	Mebhydroline	Tablet 50 mg	50–100 mg, 2–3 times/day	Well absorbed. Metabolised in liver.	Allergic conditions	
Oxatomide	Tinset	Tablet 30 mg	30 mg twice a day	Well absorbed. T_{max} 2–4 h. Metabolised in liver. $T_{1/2el}$ = 14 h.	Allergic conditions	
Promethazine	Phenergan Promethazine	Injection liquid 2 mg/mL, 1 mL or 2 mLTablet 25 mg Syrup 1 mg/mL	25–150 mg/day	Well absorbed. T_{max} 20 min, duration 6–12 h. Metabolised in liver. $T_{1/2el}$ = 10–14 h.	Allergic conditions/ anaphylactic shock	Acute asthma attack, sensitivity to phenothiazines
Acrivastine	Semprex	Capsule 8 mg	8 mg/day	Well absorbed. T_{max} 2 h, duration 4–6 h. Metabolised in liver. $T_{1/2el}$ = 20 h.	Allergic rhinitis / hayfever	Triprolidine sensitivity

Astemizole	Hismanal	Tablets 10 mg Suspension 10 mg/5 ml	10 mg/day		Urticaria	
Cetirizine	Reactine Zyrtec	Drink 1 mg/mL Tablet 10 mg	10 mg/day	Well absorbed. T_{max} 60–90 min. Metabolised in liver. $T_{1/2el}$ = 10 h.	Allergic rhinitis/ conjunctivitis/ urticaria	Preservative sensitivity
Fexofenadine	Telfast	Tablet 120 mg, 180 mg	120 mg/day rhinitis 180 mg/day urticaria	Well absorbed. T_{max} 1–3 h. Not metabolised. $T_{1/2el}$ = 11–15 h.	Allergic rhinitis/ chronic urticaria	
Ketotifen	Zaditen	Tablet 1 mg Syrup 0.2 mg/mL Drops 1 mg/mL	1–2 mg/day	Well absorbed. T_{max} 2–4 h, duration 4–6 h. Metabolised in liver. $T_{1/2el}$ = 22 h.	Allergic rhinitis/ allergic skin conditions/ prophylactic for asthma	Preservative sensitivity
Levocabastine	Livocab nose drops and eye drops Livostin	0.5 mg/mL	Spray twice, twice a day 1 drop per eye twice a day	Well absorbed. Not metabolised. $T_{1/2el}$ = 35–40 h.	Allergic rhinitis/ conjunctivitis	Preservative sensitivity
Levocetirizine	Xyzal	Tablet 5 mg	5 mg/day	Well absorbed. T_{max} 1 h. Partially metabolised in liver. $T_{1/2el}$ = 6–10 h.	Allergic rhinitis/ chronic urticaria	Kidney insufficiency, lapp-lactase-deficiency, glucose-galactose malabsorption
Terfenadine	Triludan Terfenadine Seldane	Suspension 6 mg/mL Tablet 60 mg	60–120 mg/day	Well absorbed. T_{max} 1–3 h. Not metabolised. $T_{1/2el}$ = 11–15 h.	Allergic rhinitis/ conjunctivitis / allergic skin disorders	Disturbed liver or heart function
Azelastine	Allergodil Astelin	Nose spray 1 mg/mL Eye drops 0.5 mg/mL	Use spray twice a day Use drops twice a day	Well absorbed. Duration 12 h. Metabolised in liver. $T_{1/2el}$ = 20–45 h.	Allergic rhinitis/ conjunctivitis	Preservative sensitivity
Desloratidine	Aerius	Tablet 5 mg Syrup 0.5 mg/mL	5 mg/day	Metabolised in liver. $T_{1/2el}$ = 27 h.	Allergic rhinitis	

Appendix C3. Marketed anti-allergic products (continued)

Chemical name	Trade name	Formulation	Usual dose	Pharmacokinetics	Indications	Contra-indications
Ebastine	Kestine Ebastel	Tablet 10 mg	10 mg/day	Well absorbed. T_{max} 1 h, duration >48 h. $T_{1/2el}$ = 15–19 h.	Allergic rhinitis/ conjunctivitis	Liver insufficiency
Loratadine	Allerfre Claritin	Tablet 10 mg Syrup 1 mg/mL	10 mg/day	Well absorbed. Metabolised in liver. $T_{1/2el}$ = 12 h.	Allergic rhinitis/ conjunctivitis/ chronic urticaria/ pruritis	Preservative sensitivity
Mizolastine	Mistalin Mizollen	Tablet 10 mg	10 mg/day	Well absorbed. T_{max} 1–2 h, duration 24 h. Metabolised in liver. $T_{1/2el}$ = 13 h.	Allergic rhinitis/ conjunctivitis/ urticaria	Disturbed liver or heart function
Monoclonal antibody						
Anti-IgE	Omalizumab Xolair	Injection liquid	150–375 mg every 2–4 weeks		Moderate to severe asthma	Urticarial rash
Anti-leukotrienes						
Montelukast	Singulair	Tablet 10 mg Chewable tablet 4 and 5 mg Granules 4 mg	>15 yrs, 10 mg/ day 6–14 yrs, 5 mg/ day 1–5 yrs, 4 mg/ day	Well absorbed. T_{max} 1.7 h. Metabolised in liver. $T_{1/2el}$ = 2.7–5.5 h.	Chronic asthma; seasonal allergic rhinitis	Children under 1 yr Not indicated for acute broncho-spasm or in pregnant women
Zileuton	Zyflo	Tablet 600 mg	600 mg, 4 times/day	Rapidly absorbed. T_{max} 3–4 h. Metabolised in liver. $T_{1/2el}$ = 2.5 h.	Chronic asthma	Active liver disease or transaminases 3× upper limit. Not indicated for acute bronchospasm

Appendix C6. Licensed cytokine therapeutics

Drug	Corporation	Indication	Approval
Actimmune® IFN-γ-1b	InterMune Pharmaceuticals	Management of chronic granulomatous disease	December 90
Actimmune® IFN-γ-1b	InterMune Pharmaceuticals	Osteopetrosis	February 2000
Alferon N Injection® IFN-α-n3 (human leukocyte derived)	Interferon Sciences	Genital Warts	October 89
Betaseron® RECOMBINANT IFN-β-1b	Berlex Laboratories	Relapsing, remitting multiple sclerosis	July 93
BMP-2, bone morph. protein-2	Medtronic Sofamor Danek	Treatment of spinal degenerative disc disease	July 02
Denosumab	Genentech	Treatment and prevention of Osteoporosis (Europe)	December 09
Enbrel® TNFR:Fc	Amgen	Active ankylosing spondylitis	July 03
Enbrel® TNFR:Fc	Immunex	Moderate to severe active rheumatoid arthritis	November 98
Enbrel® TNFR:Fc	Immunex	Moderate to severe active juvenile rheumatoid arthritis	May 99
EPOGEN® Epetin α (rEPO)	Amgen	Anemia caused by chemotherapy	April 93
EPOGEN® Epetin α (rEPO)	Amgen	Anemia, chronic renal failure, anemia in Retrovir® treated HIV-infected	June 89
EPOGEN® Epetin α (rEPO)	Amgen	Chronic renal failure, dialysis	November 99
EPOGEN® Epetin α (rEPO)	Amgen	Surgical blood loss	December 96
Erythropoiesis stimulating agent darbepoetin Alfa	Roche	Stimulating agent	2001
Infergen® IFN alfacon-1	Amgen	Treatment of chronic hepatitis C viral infection	October 97
Intron® A IFN-α-2b	Schering-Plough	AIDS-related Kaposi's sarcoma	November 88
Intron® A IFN-α-2b	Schering-Plough	Follicular lymphoma	November 97
Intron® A IFN-α-2b	Schering-Plough	Follicular lymphoma	November 97
Intron® A IFN-α-2b	Schering-Plough	Genital warts	June 88

Appendix C6. Licensed cytokine therapeutics (continued)

Drug	Corporation	Indication	Approval
Intron® A IFN-α-2b	Schering-Plough	Hairy cell leukemia	June 86
Intron® A IFN-α-2b	Schering-Plough	Hepatitis B	July 92
Intron® A IFN-α-2b	Schering-Plough	Hepatitis C	February 91
Intron® A IFN-α-2b	Schering-Plough	Malignant melanoma	December 95
Leukine™ sargramostim (GM-CSF)	Immunex	Allogeneic bone marrow transplantation	November 95
Leukine™ sargramostim (GM-CSF)	Immunex	Autologous bone marrow transplantation	March 91
Leukine™ sargramostim (GM-CSF)	Immunex	Neutropenia resulting from chemotherapy	September 95
Leukine™ sargramostim (GM-CSF)	Immunex	Peripheral blood progenitor cell mobilization	December 95
LFA-1/IgG1	Biogen	Moderate to severe chronic plaque psoriasis	January 03
Mircera® (methoxy polyethylene glycol beta)	Roche	Anemia associated with chronic renal failure	November 07
Neumega®, oprelvekin rHu IL-11	Genetics Institute	Chemotherapy-induced thrombocytopenia	November 97
NEUPOGEN® Filgrastim (rG-CSF)	Amgen	Acute myeloid leukemia	April 98
NEUPOGEN® Filgrastim (rG-CSF)	Amgen	Autologous or allogeneic bone marrow transplantation	
NEUPOGEN® Filgrastim (rG-CSF)	Amgen	Chemotherapy-induced neutropenia	February 91
NEUPOGEN® Filgrastim (rG-CSF)	Amgen	Chronic severe neutropenia	December 94
NEUPOGEN® Filgrastim (rG-CSF)	Amgen	Peripheral blood progenitor cell transplantation	December 95
Nplate romiplostian peptide agonist for TPO-R	Amgen	Treatment of thrombocytopenia in patients with idiopathic thrombocytopenia purpura	August 08
Ontak Diphtheria Tox – IL-2	Sevagen	CTCL	February 99
Proleukin® aldesleukin (IL-2)	Chiron	Metastatic melanoma	
Proleukin® aldesleukin (IL-2)	Chiron	Renal cell carcinoma	May 92

Rebetron™ ribavirin/IFN-α-2b	Schering-Plough	Chronic hepatitis C	June 98
Rebetron™ ribavirin/IFN-α-2b	Schering-Plough	Chronic hepatitis C, compensated liver disease	December 99
Rebif® IFN-β-1a	Serono	Relapsing forms of multiple sclerosis	March 02
Regranex® Becaplermin, rHPDGF-BB	Ortho-McNeil	Diabetic neuropathy, foot ulcers	December 97
Roferon® IFN-α-2a	Hoffman-La Roche	AIDS-related Kaposi's sarcoma	November 88
Roferon® IFN-α-2a	Hoffman-La Roche	Chronic myelogenous leukemia	November 95
Roferon® IFN-α-2a	Hoffman-La Roche	Hairy cell leukemia	June 86
Roferon® IFN-α-2a	Hoffman-La Roche	Hepatitis C	November 96
Stemgen (stem cell factor)	Amgen	Mobilization (Australia, New Zealand, Canada)	1997
Wellferon® IFN-n	Glaxo Wellcome	Treatment of hepatitis C in patients 18 years-of-age or older without decompensated liver disease	March 99

Appendix C7. Examples of immunomodulators marketed as drugs/therapeutics for microbial infections

Name	Trade name	Dose	Pharmacokinetics	Indications	Contraindications
IFN-α2b	Intron A	5–10 million units s.c./i.m. 3×/wk ×16 wk for hepatitis B, 3 million units × 24–48 wk for hepatitis C in combination with Ribavirin	Metabolized in kidney, excreted in urine, half-life 2–3 h	Chronic hepatitis B and C	Hypersensitivity to IFN, autoimmune hepatitis, severe depression, psychiatric disorder
PegIFN-α2b	Peg-Intron	Monotherapy with 0.0–1 μg/kg/wk s.c., 1.5 μg/kg/wk s.c. in combination with Ribavirin	Excreted in urine, half-life 40 h	Chronic hepatitis C	Hypersensitivity to IFN, autoimmune hepatitis, severe depression, psychiatric disorder
PegIFN-α2a	Pegasys	135–180 μg s.c. 24–48 wk for hepatitis B, for hepatitis C in combination with Ribavirin	Excreted in urine, half-life 80 h	Chronic hepatitis B and C	Hypersensitivity to IFN, autoimmune hepatitis
IFN-β	Fiblaferon	5–10 million units	Metabolized in liver, half-life 8 min–4 h	Viral encephalitis, herpes zoster varicella in immunosuppressed patients	Hypersensitivity to IFN or albumin, depression
IFN-γ1b	Imukin	2 million units	Half-life 38 min–5.9 h	Chronic granulomatous disease	Hypersensitivity to IFN, cardiac patients, liver and kidney insufficiency
G-CSF/ Filgrastim	Neupogen	1–10 μg/kg s.c.	Half-life 3.5 h	AIDS neutropenia	Hypersensitivity to CSF or *E. coli* proteins
Pegfilgrastim	Neulasta	6 mg s.c.	Half-life 15–80 h	Neutropenia	Hypersensitivity to CSF or *E. coli* proteins
GM-CSF	Sargra-mostim	250 μg i.v./s.c.	Half-life 60 min i.v., 162 min s.c.	Neutropenia	Hypersensitivity to CSF, concomitant chemotherapy
Maraviroc	Selzentry	150–300 mg	Metabolized in liver, half-life 14–18 h	CCR5-tropic HIV	Hypersensitivity to drug, CXCR4-tropic HIV
Imiquimod	Aldara	5% 3×/wk	Excreted in urine and feces	Condyloma acuminata	Hypersensitivity to drug, avoid exposing to UV light and sun

Isoprinosine	Delimmun	Dimepranolacedoben 379.75 mg inosine 120.25 mg	Excreted in urine, half-life 3.5 h for DIP and 50 min for PAcBA	Herpes simplex infections, acute viral encephalitis	Hypersensitivity to drug, elevated uric acid, pregnancy
Thalidomide	Thalomid	100–300 mg PO	Excreted in urine, half-life 5–7 h	HIV wasting	Hypersensitivity to drug, pregnancy
Bacterial extract	Broncho-Vaxom	3.5–7 mg lyophilized bacterial extract	Not known	Recurrent infections of the airways	Acute gastrointestinal infection, autoimmune disease
	Luivac	3 mg lysate	Not known	Recurrent infections of respiratory tract	Acute gastrointestinal infection, autoimmune disease

Appendix C9. Immunomodulatory phytotherapeutics and dietary supplements available commercially

Chem. name	Trade name	Formulation	Usual dose	Pharmacokinetics	Indications	Contraindications
Echinacea purpurea (purple coneflower)	Echinacin, Echinagard, Echinaforce, Esberitox Mono	Extract as tincture or pastilles	60-80 g squeezed sap per 100 g	Alkylamides detected in blood within 10 min (probably buccal absorption) after oral administration of extract, showing linear dose-dependency; t_{max} = 30 min (1–2 h at higher extract concs.). $T_{1/2}$ = ca. 1 h. Potential interactions with CYPs 3A4, 1A2, 2C9	Adjuvant therapy of respiratory infections; wound healing	Hypersensitivity to Echinacea
Viscum album (mistletoe)	Iscador, Helixor, Eurixor, Lektinol, Lentinan	Extract for intracutaneous injection (Europe only)	1 ng/kg ML-I equiv. s.c.	ML-I crosses cell membrane to induce cytotoxicity	Adjuvant therapy of cancer	
Zinc sulphate	Solvezink	Tablets	Zinc sulphate 200 mg, equiv. to elemental zinc 45 mg	Zinc sulphate is partially absorbed from the gastrointestinal tract, distributed in the skeletal muscle, skin, bone, and pancreas, and excreted primarily through the duodenum and jejunum.	Zinc salts are primarily used to relieve minor eye irritations. They have been used to correct zinc-deficiency conditions (e.g. acrodermatitis enteropathica) and other diseases associated with zinc-depletion (e.g. anorexia nervosa, arthritis, diarrhoeas, eczema, recurrent infections, and recalcitrant skin problems).	Oral zinc preparations may cause gastrointestinal upset; ophthalmic zinc preparations should be used cautiously in persons with glaucoma.
	Zincol Zixol Oral-Z	Syrup oral	Zinc sulphate, 10 mg/5 mL zinc sulphate, 1%			
	Colirio, Honfar	Drops ophthalmic	25–50 mg daily			
	Erectol	Capsules	Zinc sulphate, 220 mg		To prolong sexual activity and increase the production of semen	

Zinc gluconate	Zigg-eeze Zinc cold treatment Pharmezinc Amplified Zinc	Lozenges lozenges oral topical tablets Tablets	Zinc gluconate equiv. to zinc element, 60 mg zinc gluconate, 10 mg		Conditions requiring zinc supplementation	
Zinc picolinat	Zinc picoloinate	Tablets	25 mg			
Zinc monomethionine	Optizinc	Capsules	30 mg of zinc per capsule	Independent human and animal studies show that zinc methionine exhibits a superior absorption rate when compared with many other forms of zinc. OptiZinc provides a superior source of bioavailable zinc. OptiZinc resists binding with dietary fibre and phytic acid.	Suitable for individuals with malabsorption and poor zinc status. Suitable for vegetarians and vegans.	Supplemental zinc can inhibit the absorption and availability of copper. Zinc monomethionine is not recommended for those person showing hypersensitivity to zinc and/or methionin.
Sodium selenite	Selemun Selen Loges	Tablets film-coated Tablets Oral unit dose liquids	46.7 µg Tablets 300 µg Se Oral unit dose liquids 100 µg Se		Selenium deficiency	
Selenomethionine	Sethotope				Contrast medium radiography	

Appendix C9. Immunomodulatory phytotherapeutics and dietary supplements available commercially (continued)

Chem. name	Trade name	Formulation	Usual dose	Pharmacokinetics	Indications	Contraindications
Seleno-yeast	Selenium yeast	Tablets	50–300 µg	Absorption of Se from single dose ^{77}Se-enriched yeast (in subjects stabilized for 10 weeeks on 300 µg Se/day) i= 89%; retention = 74%. Plasma C_{max} = 9.8 µg/L; T_{max} = 9.2 h: $T_{1/2}$ = 1.7 days for initial phase (0.5–2 days), 3 days for middle phase (2–3 days) and 11.1 days for later phase (3–14 days). ^{77}Se excretion = 47 µg in urine and 37 µg in faeces.	Selenium deficiency	
Ascorbic acid (vitamin C)	Ascorbex, Ascorbic Acid Injection BP 2002, Ascorbic Acid Tablets BP 2002, Balanced C Complex, C-Vit, C-Will, C-vimin, Cebiolon, Cebion, Cecap, Cecon, Cecrisina, Ceerexin, Celin, Cemina, Cereon, Cetebe, Cetrinets, Cevalin, Cevi-Bid, Cevi-Tablets, Cevidrops, Cevita, Cevitol, Cewin, Citron, Citrovit, Dancimin-C,	Tablets, effervescent, injections	In deficiency states: oral or intramuscular doses of 100–500 mg/day; for urine acidification: 3–12 g/day as divided doses every 4 h; prophylactic oral or intramuscular doses for paediatric patients: 30–60 mg/day (age-dependent); paediatric patients with scurvy:100 mg 3 times a day for 1 week	Peak serum levels: 2–3 h after oral dosing; therapeutic serum levels: 0.4–1.5 mg/dL; without supplementation 75 mg of ascorbic acid is excreted in the urine daily, increasing to 400 mg within 24 h with the administration of 1 g day; haemodialysis and peritoneal dialysis remove significant amounts of the drug and supplementation is suggested following dialysis periods.	An essential vitamin for deficiency states. Also used in large doses as a urine acidifier for patients with urinary tract infections. Requirements for ascorbic acid may increase during infection, trauma, pregnancy, and lactation.	Adverse effects include haemolysis, oesophagitis, intestinal obstruction, hyperoxaluria, renal failure, and injection site pain.

Ascorbic acid (vitamin C) (continued)	Delrosa, Demovit C, Energil C, Eriglobin, Eritropiu, Grad vitamine C, Hermes Cevitt, Hicee, Ido-C, Kendural C, Laroscorbine, Megafer, Megavit C 1000, Midy Vitamine C, Nutrol C, Pore-max-C, Rovit C, Scorbex, Sidervim, Super C, Tetesept Vitamin C, Timed Release C, Upsavit C, Vicemex, Vicomin C		and 100 mg daily thereafter until clinical symptoms abate.			
α-Tocopherol (vitamin E)	Alfa-E, Aqua-sol-E, Bio E, Bio-E-Vitamin, Biogenis Biop-to-E, Biosan E 600 Burger-stein, Vitamin E Dalfarol, Dif-Vitamin E, E 200/400, E-Tab, E-Vitamin-ratiopharm, E-Vitamin, Malton E,	Capsules, injections	100 mg/kg/day orally; paediatric doses range from 1 mg/kg/day to 100 mg/day.	Oral absorption from the gastrointestinal tract is variable; bile is necessary. Water-miscible forms are absorbed intramuscularly; oil preparations not as well. Vitamin E is distributed to all tissues and is predominately metabolized in the liver. The half-life after intramuscular injection is 44 h and after intravenous injection about 282 h.	Vitamin E is useful in many conditions including malab-sorption diseases, haematological dis-eases, cardiovascular disease, and retrolen-tal fibroplasia.	Vitamin E is gener-ally well-tolerated and enhances the response to oral anticoagulants. Fatigue, weak-ness, headache, nausea, diarrhoea, flatulence and abdominal pain, and delayed coag-ulation times in vitamin-K deficient patients may occur with large doses.

Appendix C9. Immunomodulatory phytotherapeutics and dietary supplements available commercially (continued)

Chem. name	Trade name	Formulation	Usual dose	Pharmacokinetics	Indications	Contraindications
α-Tocopherol (vitamin E) (contiued)	Mediderm, Natural Made Vitamin E, One-A-Day Extras E, Opto-vit E, Opto-vit, Plenovit, Tocorell Vit. E, Togasan Vitamin E, Topher-E, Unique E					Major signs of toxicity include fatigue and weakness. Topical vitamin E has rarely caused contact dermatitis and erythema multiforme.
Epigallocatechin gallate (EGCG)	Tea catechin, Polphenon E	Tablets, capsules	As green tea extracts; also standardized for EGCG content (up to 2000 mg bid)	EGCG is readily detected in human and rat plasma at ng/mL levels following oral ingestion	Lipid-lowering agent	
Decaffeinated green tea polyphenol mixture	Polyphenon E	Ointment, tablets		Steady-state plasma EGCG levels range from 2.9 ng/mL (400 mg bid p.o.) to 3.974 ng/mL (2000 mg bid p.o.) after 1 month daily administration.	Treatment of genital warts, intended oral treatment of chronic lymphocytic leukaemia.	

Source: Lifecycle (IMS), Micromedex (International Health Series, Martindale, Index Nominum), PharmaProjects, PubMed

Appendix C10. Antibacterial agents currently in use for their potential* immunomodulatory properties
Erythromycin A
Clarithromycin
Azithromycin
Roxithromycin
Minocycline
Doxycycline
Rifamycin SV
Dapsone
Clofazimine

**The mechanism (immunomodulatory or antibacterial) underlying the therapeutic benefit still remains controversial for some clinical settings*

Appendix C14/1. Structures and properties of non-steroidal anti-inflammatory drugs

Drug	Chemical name	Trade name	Formulation	Usual dose	Pharmacokinetics	Indications	Contraindications
Aspirin	Acetylsalicylic acid	Angettes, Caprin, Nu-seals aspirin	Tablets	75mg/day, or 300 mg 3 or 4 times daily	Highly bound in plasma, systemic action only at normal doses	Pain, rheumatism, antiplatelet therapy	Peptic ulcers, children under 12, asthma, anticoagulants, hypoglycaemic agents
Diflunisal	5-(2,4-difluorophenyl) salicylic acid	Dolobid	Tablets	500–1000 mg daily	Highly bound in plasma, systemic action only at normal doses	Pain, arthritis	Peptic ulcers, asthma, renal impairment, anticoagulants, pregnancy, lactation
Ibuprofen	2-(4-isobutylphenyl)-propionic acid	Brufen, Nurofen, Motrin, Ext: Ibugel, Fenbid gel.	Tablets, topical preparations	1200–1800 mg daily in divided doses	Highly bound in plasma, systemic action only at normal doses	Pain, pyrexia, arthritis,	Peptic ulcer, asthma, renal impairment, anticoagulants, pregnancy, lactation
Naproxen	d-2-(6-methoxy-2-naphthyl)propionic acid	Naprosyn, Nycopren, Synflex	Tablets	250–500 mg twice daily	Highly bound in plasma, systemic action only at normal doses	Arthritis, musculoskeletal disorders, dysmenorrhoea	Peptic ulcer, asthma, renal impairment, anticoagulants
Fenoprofen	α-dl-2-(3-phenoxy-phenyl) propionic acid	Fenopron	Tablets	300–600 mg 3–4 times daily	Highly bound in plasma, systemic action only at normal doses	Pain, arthritis, musculoskeletal disorders	Peptic ulcer, asthma, renal impairment, anticoagulants, hypoglycaemics
Fenbufen	3-(4-biphenylylcarbonyl)propionic acid	Lederfen	Tablets and capsules	450 mg twice daily	Highly bound in plasma, only systemic action at normal doses	Arthritis, musculoskeletal disorders	Peptic ulcer, asthma, renal impairment, anticoagulants

Flurbiprofen	2-(2-fluoro-4-biphenylyl)-propionic acid	Froben, Ocufen, Streflam	Tablets and suppositories, eye drops, throat lozenges	150–200 mg daily, up to 300 mg daily	Highly bound in plasma, only systemic action at normal doses	Arthritis, musculoskeletal disorders, post operative analgesia, sore throat	Peptic ulcer, asthma, renal impairment, anticoagulants, pregnancy, lactation
Ketoprofen	2(3-benzoylphenyl)propionic acid	Ketocid, Orudis, Oruvail, Oruvail gel, Powergel	Capsules, suppositories, injection or gel	100–200 mg once daily with food	Highly bound in plasma, only systemic action at normal doses	Arthritis, musculoskeletal disorders, after orthopaedic surgery, dysmenorrhoea	Peptic ulcer, asthma, renal impairment, anticoagulants, not children, pregnancy, lactation
Tiaprofenic acid	α-methyl-5-benzoyl-2-thienylacetic acid	Surgam	Tablets, capsules	600 mg daily in 2–3 divided doses	Highly bound in plasma, only systemic action at normal doses	Arthritis and other musculoskeletal disorders	Peptic ulcer, asthma, renal impairment, anticoagulants
Indomethacin	1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-3-indolyl-acetic acid	Indocid, Indomod, Flexin Continus, Indocid PDA	Capsules, suppositories, tablets, injection	50–200 mg daily, i.v. infusion over 20–30 mins	Highly bound in plasma, central effects e.g. headache, vertigo, mental confusion, depression	Arthritis, musculoskeletal disorders, dysmenorrhoea, patent ductus arteriosus	Peptic ulcer, asthma, aspirin allergy, renal impairment, pregnancy, anticoagulants
Sulindac	Cis-5-fluoro-2methyl-1-[p-methylsulphinyl) benzylidene] indene-3-acetic acid	Clinoril	Tablets	200 mg twice daily	Highly bound in plasma, prodrug for sulindac sulphide	Arthritis, peri-articular disorders, musculoskeletal disorders	Peptic ulcer, asthma, renal impairment, anticoagulants, not children
Ketorolac	(±)-5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid	Toradol, Acular,	Injection, eye drops	10–30 mg every 4–6 h	Highly bound in plasma, only systemic action	Post-operative pain	Peptic ulcer, asthma, renal impairment
Tolmetin	5-(*p*-toluoyl)-1-methylpyrrole-2-acetic acid	Reutol, Tolectin, Tolmene	Tablets	0.8–1.6 g/day	Highly bound in plasma, only systemic action	Rheumatoid and osteoarthritis	Peptic ulcer, anticoagulants, hypoglycaemics

Appendix C14/I. Structures and properties of non-steroidal anti-inflammatory drugs (continued)

Drug	Chemical name	Trade name	Formulation	Usual dose	Pharmacokinetics	Indications	Contraindications
Diclofenac	2-[(2,6-Dichlorophenyl)amino] benzene acetic acid	Dicloflex, Diclomax, Motifene, Voltarol, Voltarol Emulgel, Volsaid Retard	Tablets, capsules, formulation with misoprostol (Arthrotec)	75–150 mg daily in 2 or 3 divided doses	Highly bound in plasma, only systemic action at therapeutic doses	Arthritis, musculoskeletal disorders, migraine, postoperative pain, dysmenorrhoea	Peptic ulcer, asthma, renal insufficiency, pregnancy, anticoagulants, hypoglycaemics
Aceclofenac	2-[(2,6-dichlorophenyl)-amino] phenylactoxyacetic acid	Preservex	Tablets	100 mg twice daily	Highly bound in plasma, only systemic action	Rheumatoid and osteoarthritis	Peptic ulcer, renal impairment, pregnancy, antidiabetics
Azapropazone	5-(Dimethyl amino)-9-methyl-2-propyl-1*H*-pyrazolo[1,2-α][1,2,4]benzotriazine-1,3(2*H*)-dione	Rheumox	Tablets	1–2 g daily in 2 or 4 doses	Highly bound in plasma, only systemic action	Rheumatoid arthritis, acute gout	Peptic ulcer, renal impairment, asthma, pregnancy
Piroxicam	4-Hydroxy-2methyl-*N*-2-pyridinyl-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide	Brexidol, Feldene, Feldene gel (ext.)	Tablets, capsules	20 mg per day	Highly bound in plasma, only systemic action	Arthritis, musculoskeletal disorders, postoperative pain	Peptic ulcer, renal impairment, asthma, anticoagulants
Tenoxicam	4-Hydroxy-2-methyl-*N*-2-pyridinyl-2*H*-thienol[2,3-e]-1,2-thiazine-3-carboxamide 1,1-dioxide	Mobiflex	Tablets	20 mg per day	Highly bound in plasma, only systemic action	Arthritis, soft tissue injuries	Peptic ulcer, renal impairment, anticoagulants

Mefenamic acid	2-[(2,3-Dimethylphenyl)amino] benzoic acid	Ponstan	Tablets	500 mg 3 times daily	Highly bound in plasma	Arthritis, dysmenorrhoea	Peptic ulcers, renal impairment, anticoagulants
Nabumetone	4-(6-Methoxy-2-naphthalenyl)-2-butanone	Relifex	Tablets	2×500 mg at bedtime	Prodrug for 6-methoxy-2-naphthylacetic acid	Rheumatoid and osteoarthritis	Peptic ulcer, renal impairment, pregnancy, anticoagulants
Etodolac	1,8-Diethyl-1,3,4,9-tetrahydropyrano-[3,4-b] indole-1-acetic acid	Lodine	Tablets	600 mg daily	Highly bound in plasma	Rheumatoid and osteoarthritis	Peptic ulcer, renal impairment, pregnancy, anticoagulants
Meloxicam	4-Hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide	Mobic	Tablets, suppositories	7.5–15 mg daily	Highly bound in plasma, CNS effects	Rheumatoid and osteoarthritis	Peptic ulcer, renal impairment, not children, anticoagulants
Nimesulide	N-(4-Nitro-2-phenoxyphenyl) methanesulphonamide	Aulin, Mesulid, Nimed	Tablets, powder	100 mg twice daily	Highly bound in plasma, short half-life	Osteoarthritis, musculoskeletal disorders, dysmenorrhoea, post-operative pain	Peptic ulcer, liver insufficiency, asthma, allergic reactions to NSAIDs, anticoagulants
Celecoxib	(4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl] benzenesulphonamide	Celebrex	Capsules	200–400 mg daily	Highly bound in plasma, crosses blood brain barrier	Osteoarthritis, rheumatoid arthritis	Peptic ulcer, renal impairment, pregnancy, hypersensitivity
Rofecoxib	4-(4′-methylsulphonylphenyl)-3-phenyl-2,5*H*)-furanone	Vioxx	Tablets	12.5–25 mg once daily	Moderately bound in plasma, crosses blood brain barrier	Osteoarthritis, rheumatoid arthritis, acute pain	Peptic ulcer, renal impairment, heart failure, pregnancy

Appendix C14/1. Structures and properties of non-steroidal anti-inflammatory drugs (continued)

Drug	Chemical name	Trade name	Formulation	Usual dose	Pharmacokinetics	Indications	Contraindications
Etoricoxib	(5-chloro-2-(6-methylpyridine-3-yl)-3-(4-methyl-sulphonylphenyl) pyridine	Arcoxia Algix Tauxib	Tablets	60–120 mg once daily	Highly bound in plasma, crosses blood brain barrier	Osteoarthritis, rheumatoid arthritis, gouty arthritis	Peptic ulcer, renal impairment, pregnancy, heart failure
Valdecoxib	4-[5methyl-3-phenyl-oxisol-4-yl]-benzene sulphonamide	Bextra	Tablets	10–40 mg once daily	Interacts with inducers or inhibitors of cytochrome P450	Osteoarthritis, rheumatoid arthritis, dysmenorrhoea	Peptic ulcer, renal impairment, hypersensitivity, anticoagulants
Parecoxib	(*N*-2[[(5-methyl-3-phenyloxisol-4yl)-phenyl]sulphonyl]propane amide	Dynastat	Injection	40 mg i.v. or i.m.	Interacts with inducers or inhibitors of cytochrome P450	Post-operative pain	Peptic ulcer, renal impairment, pregnancy, anticoagulants
Lumiracoxib	(2-[6-chloro-6-fluorophenyl) amino]-5-methylphenyl)acetic acid	Prexige	Tablets	100–400 mg once daily	Highly protein bound in plasma. Hepatic metabolism. Half life 4h.	Osteoarthritis, dysmenorrhoea, acute and post-operative pain	Hepatic or renal impairment. Withdrawn in November 2007
Paracetamol	*N*-(4-Hydroxy-phenyl)acetamide	Alvedon, Calpol, Infadrops, Medinol	Suppositories, suspension, drops, tablets	60–250 mg, 500 mg.	Crosses blood brain barrier	Mild to moderate pain and pyrexia	Renal or hepatic impairment

Appendix C14/II. Marketed NSAIDs

Class I. Non-selective COX-1 and COX-2 inhibitors

Salicylates
Sodium salicylate
Aspirin
Diflunisal

Propionic acid derivatives
Ibuprofen
Naproxen
Fenbufen
Fenoprofen
Flurbiprofen
Ketoprofen
Tiaprofenic acid

Acetic acid derivatives
Indomethacin
Sulindac
Ketorolac
Tolmetin
Diclofenac
Aceclofenac

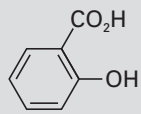
Pyrazolones
Azapropazone
Phenylbutazone

Oxicams
Piroxicam
Tenoxicam

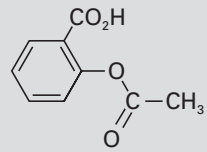
Fenamic acids
Mefenamic acid
Meclofenamic acid

Nabumetone
6-methoxy-2-naphthylacetic acid (active metabolite)

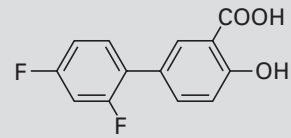
Appendix C14/II. Chemical Structures of the NSAIDs



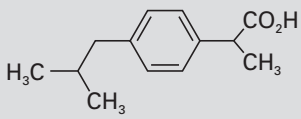
Salicylic acid



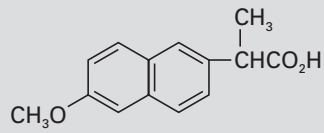
Aspirin (Acetylsalicylic acid)



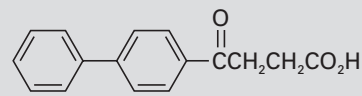
Diflunisal



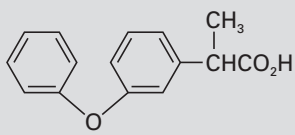
Ibuprofen



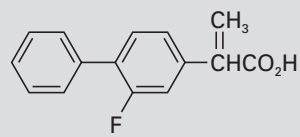
Naproxen



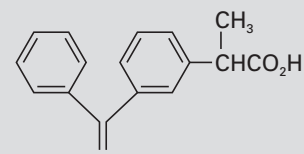
Fenbufen



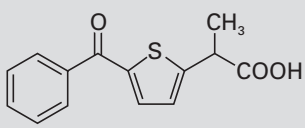
Fenoprofen



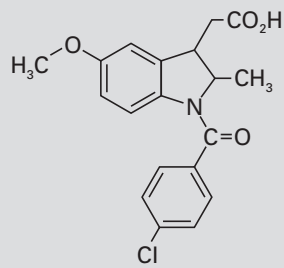
Flurbiprofen



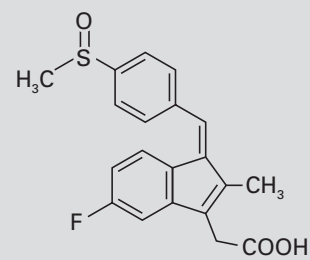
Ketoprofen



Tiaprofenic acid

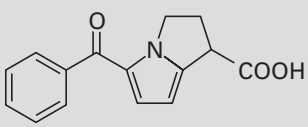


Indomethacin

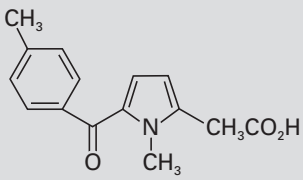


Sulindac

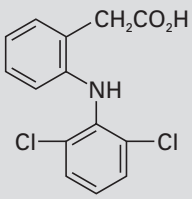
Appendix C14/II. Chemical Structures of the NSAIDs (continued)



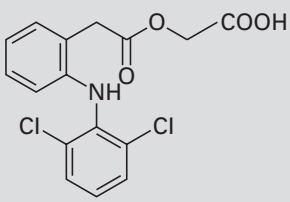
Ketorolac



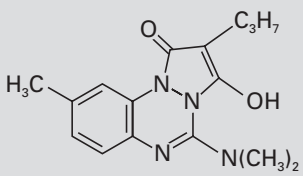
Tolmetin



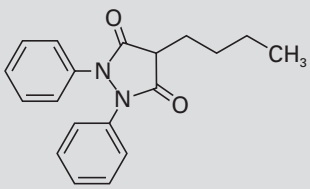
Diclofenac



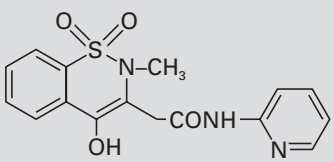
Aceclofenac



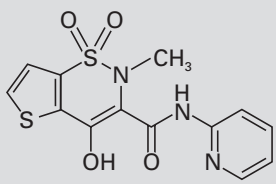
Azapropazone



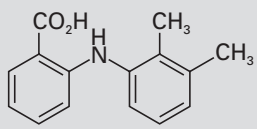
Phenylbutazone



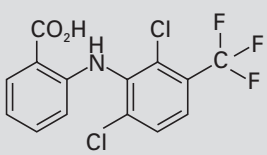
Piroxicam



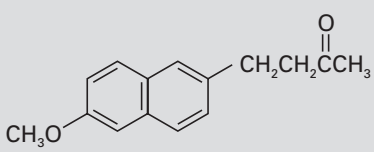
Tenoxicam



Mefenamic acid



Mefenamic acid



Nabumetone

Appendix C14/II. Marketed NSAIDs (continued)

Class I. Non-selective COX-1 and COX-2 inhibitors

Mode of action

These drugs all inhibit COX-1 and COX-2 non-selectively, but to different degrees, e.g. piroxicam and ketorolac inhibit COX-1 very much more potently than COX-2, but diclofenac and ibuprofen at therapeutic doses cause almost similar inhibition of both enzymes. They inhibit both COX-1 and COX-2 by competing with the substrate, arachidonic acid, for the active site on the enzyme. Aspirin is unusual in acetylating Ser530 in the active site and preventing access of arachidonic acid. The action of aspirin is irreversible and inhibition of COX-1 in platelets is responsible for the antithrombotic activity of aspirin.

Pharmacological effects

These drugs all have analgesic, antipyretic and anti-inflammatory effects.

Cellular pharmacokinetics

The inhibitory actions on COX enzymes are mainly peripheral as these drugs are acidic, polar compounds and cross the blood-brain barrier with difficulty. However, toxic doses may cause central symptoms, e.g. salicylate and aspirin in high doses cause dizziness and tinnitus. These acidic drugs are also highly bound to plasma proteins (95–99%) which leads to interactions with other highly bound drugs such as anticoagulants.

Clinical indications

The main therapeutic use is for pain and inflammation in rheumatic diseases such as rheumatoid arthritis and osteoarthritis; also in various other musculoskeletal disorders. Daily low doses of aspirin are administered for their antiplatelet effects.

Side effects

a) The major side effect and disadvantage to the use of non-steroidal anti-inflammatory drugs is damage to the gastric mucosa resulting in ulceration and bleeding. This is due to removal of the gastroprotective prostaglandins synthesized by COX-1 in the stomach wall. Drugs which are highly selective for COX-1 over COX-2, such as piroxicam, cause greater incidence of bleeding than drugs such as diclofenac or ibuprofen which are almost equipotent for COX-1 and COX-2. Arthrotec is a formulation of diclofenac with the PGE_1 analogue, misoprostol, to prevent gastric mucosal damage.
b) COX-1 and COX-2 inhibitors other than aspirin have antithrombotic actions at therapeutic dose levels but aspirin is the most effective.
c) Most of the non-selective non steroidal anti-inflammatory drugs precipitate an asthmatic episode in patients who suffer from aspirin-induced asthma.

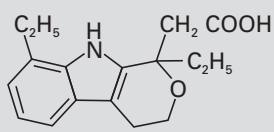
Appendix C14/II. Marketed NSAIDs (continued)

Class II. Selective COX-2 inhibitors

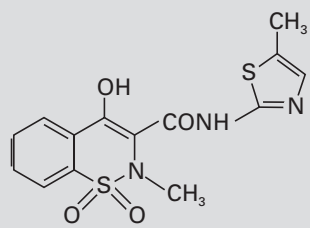
Low selectivity for COX-2
Etodolac
Meloxicam
Nimesulide

High selectivity for COX-2

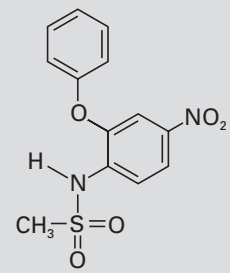
Celecoxib	Valdecoxib
Rofecoxib	Parecoxib
Etoricoxib	Lumiracoxib



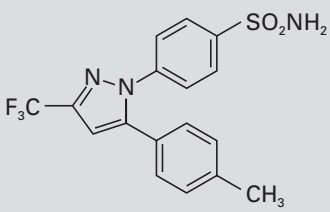
Etodolac



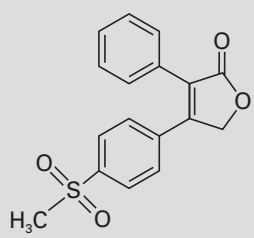
Meloxicam



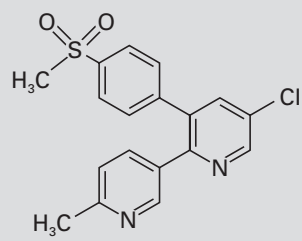
Nimesulide



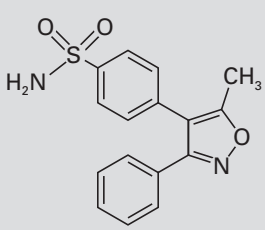
Celecoxib



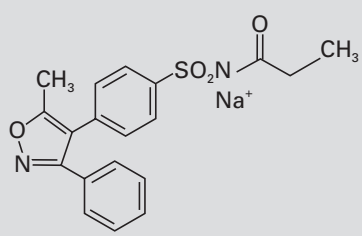
Rofecoxib



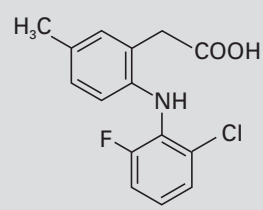
Etoricoxib



Valdecoxib



Parecoxib



Lumiracoxib

Appendix C14/II. Marketed NSAIDs (continued)

Class II. Selective COX-2 inhibitors

Mode of action

Nimesulide, celecoxib, rofecoxib, etoricoxib and valdecoxib have sulphone anilide or sulphonamide structures and are made up of larger molecules than the non-selective anti-inflammatory drugs (listed above). Parecoxib is a pro-drug of valdecoxib while lumiracoxib is a weak acid and an analogue of diclofenac. The large molecules of the selective COX-2 inhibitors do not easily fit the COX-1 active site, but comfortably occupy the larger COX-2 active site with its side pocket. Etodolac and meloxicam also have large molecules which fit more easily into the active site of COX-2 than COX-1.

Etodolac, meloxicam and nimesulide inhibit COX-2, 5–20 times more selectively than COX-1 and celecoxib, rofecoxib, etoricoxib, valdecoxib and lumiracoxib are 30–400 times more selective for COX-2 than COX-1, depending which pharmacological test is being applied.

Pharmacological effects

These drugs are analgesic, antipyretic and anti-inflammatory.

Cellular pharmacokinetics

The selective COX-2 inhibitors with sulphone anilide and sulphonamide structures cross the blood-brain barrier easily and may have central effects which have so far not been identified. They are bound to plasma proteins to a much lesser extent than the non-selective anti-inflammatory drugs (listed above) and interact to a lesser degree with other highly bound drugs such as anticoagulants.

Clinical indications

Their main therapeutic use is for the pain and inflammation of rheumatoid arthritis and osteoarthritis; also dysmenorrhoea and pain following dental surgery.

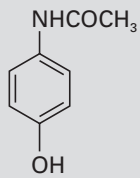
Side effects

a) The main advantage of selective COX-2 inhibitors is the sparing effect on COX-1 of the gastric mucosa. Thus, these drugs cause a much lower incidence of stomach bleeding and ulceration than the anti-inflammatory drugs of group I (listed above). However, if low dose aspirin is administered with a selective COX-2 inhibitor, the gastroprotection of the COX-2 inhibitor is largely lost. Also, celecoxib, when administered for 12 months, causes as many gastrointestinal adverse events as comparable non-selective COX-1 and COX-2 inhibitors.
b) They are sparing of COX-1 in platelets and thus have no anti-thrombotic action.
c) The incidence of renal toxic actions is no greater than with the non-selective COX-1 and COX-2 inhibitors.
d) Selective COX-2 inhibitors cause a greater incidence of myocardial infarction than some non-selective inhibitors such as naproxen or ibuprofen. However, lumiracoxib has not been shown to increase the risk of myocardial infarction.
e) Lumiracoxib has been withdrawn throughout Europe and Australia because of the risk of side effects affecting the liver.
f) Selective COX-2 inhibitors do not precipitate asthma attacks in aspirin sensitive asthma patients, confirming that aspirin-induced asthma is caused by inhibition of COX-1.

Appendix C14/II. Marketed NSAIDs (continued)

Class III. Para-aminophenol derivatives

Paracetamol (acetaminophen)



Paracetamol

Mode of action

In pharmacological tests, it is a weak inhibitor of both COX-1 and COX-2 and an inhibitory action on a third COX enzyme, COX-3 has been postulated. Alternately, the activity of paracetamol may be reduced in the presence of high levels of peroxides and this could explain its lack of anti-inflammatory action.

Pharmacological effects

Paracetamol is an antipyretic analgesic with no anti-inflammatory actions in humans.

Cellular pharmacokinetics

Paracetamol enters the central nervous system easily and its analgesic and antipyretic actions are most likely by inhibition of COX-3 in the brain and spinal cord. Plasma protein binding is equivalent to approximately 20% of the therapeutic dose.

Clinical indications

Its therapeutic use is mainly for pain of various origins such as pain of rheumatic diseases and musculoskeletal pain. The use in fever is mostly for childhood fevers and occasionally for fever of cancer or stroke patients.

Side effects

a) Paracetamol does not damage the gastric mucosa or prolong bleeding time in humans.
b) Paracetamol is safe to administer to aspirin-sensitive asthma patients.
c) The main disadvantage of paracetamol is the hepatotoxicity, which can occur at doses only slightly greater than therapeutic doses. This is caused by a metabolite of paracetamol formed by cytochrome P450, *N*-acetyl-*p*-benzoquinonimine which depletes liver glutathione and rapidly produces centrilobular necrosis. *N*-Acetyl-*p*-benzoquinonimine is inactivated by conjugation with glutathione and this reaction can be prevented by supplying sulphhydryl groups in the form of *N*-acetylcysteine. Thus, administration of *N*-acetylcysteine reverses the liver toxicity of paracetamol.

Appendix C15. Overview of DMARD used in the treatment of RA

Drug	Approximate time to benefit	Usual maintenance dose	Toxicity
Anti-TNF antibodies			Infections (all), demyelinating disorders, worsening cardiac failure, lupus-like reactions, malignancy, TB and hepatitis B/C reactivation. Injection/infusion reactions of fever, hypotension, chills, rash, local irritation.
Adalimumab	1 month	s.c. 40 mg every 2 weeks. Alone or with other DMARD.	
Infliximab	1 month	i.v. 3 mg/kg every 8 weeks. Often used with methotrexate.	
Golimumab	1 month	s.c. 50 mg monthly, i.v. 2–4 mg/kg every 3 months. Used with methotrexate	
Certolizumab	1 month	s.c. 400 mg 0, 2 and 4 weeks then 200 mg fortnightly. Used with methotrexate	
TNF receptor construct			
Etanercept	1 month	s.c. 25 mg twice a week, 50 mg weekly. Alone or with methotrexate.	Infections, injection site reactions.
IL-1 receptor antagonist			
Anakinra	1 month	s.c. 100 mg daily.	Injection site reactions.
Anti-CD20 antibody			
Rituximab	1 month	i.v. 1 g repeated after 2 weeks. Used with methotrexate.	Infusion reactions, increasing hepatitis C viral load.
Anti-IL-6 antibody			
Tocilizumab	1 month	i.v. 2–8 mg/kg every 4 weeks. Used with methotrexate	Neutropenia, elevations of plasma cholesterol and hepatic transaminases.
CD80/CD86 binding on B cell surface			
Abatacept	2–3 months	i.v. 500–1000 mg baseline and 2 weeks then every 4 weeks. Used with methotrexate	Should not be combined with etanercept.

Small molecular weight DMARD			
Methotrexate	1–2 months	Oral initial 7.5-10 mg as a single dose once a week or split into 3 doses over 36 hours. Maintenance individualized dose 15–25 mg per week	Gastrointestinal (oral ulcers, nausea, vomiting, diarrhea), CNS (headache, fatigue, fuzziness), stomatitis, rash, alopecia, rash, infections, infrequent myelosuppression, hepatotoxicity, rare but serious (even life threatening) pneumonitis.
Hydroxychloroquine	2–4 months	Oral 200–400 mg mg daily.	Infrequent rash, diarrhea, rare retinal toxicity
Leflunomide	1–4 months	Oral 10–20 mg daily. Rarely preceded with loading dose 100 mg daily for 3 days.	Diarrhea, dyspepsia, mild leukopenia, rash, alopecia, headache, dizziness. Possible severe hepatotoxicity.
Sulfasalazine	1–2 months	Oral 1000 mg 2 or 3 times daily.	Rash, infrequent myelosuppression, gastrointestinal intolerance.
Gold complexes			
Aurothiomalate	3–6 months	i.m. Initial dosage 10–25 mg once a week. Maintenance 25–50 mg every 2-4 weeks.	Rash, stomatitis, myelosuppression, thrombocytopenia, proteinuria.
Auranofin	4–6 months	Oral 3 mg once or twice daily.	Frequent diarrhea, otherwise same as aurothiomalate.

Glossary

ACCURATE: The accuracy of an assay is the degree to which it represents the true value. However, as commonly used it does not reflect the precision of the assay. Because of this some workers prefer the term bias. Accuracy is then a combination of the precision and bias of the assay.

ACETYLCHOLINE: A neurotransmitter in both the peripheral and central nervous system. In the peripheral nervous system, acetylcholine activates muscles and is a major neurotransmitter in the autonomic nervous system. In the central nervous system, acetylcholine tends to cause excitatory actions via the cholinergic system.

ACQUISITION THRESHOLD: A boundary set during flow cytometric data acquisition, below which data will not be collected by the cytometer. The boundary can be set on the basis of light scatter (forward or side scatter), fluorescence, or a combination of fluorescence and light scatter. An acquisition threshold eliminates unwanted events (e.g., debris) that might otherwise obscure collection of relevant events and/or produce excessively large files.

ACTH, CORTICOTROPIN: Hormone of the anterior lobe of the hypophysis or pituitary gland; it governs the nutrition and growth of the adrenal cortex, stimulating it to release steroid hormones, and also possesses extra-adrenal adipokinetic activity; the hormone is a polypeptide containing 39 amino acids, but exact structure varies from one species to another.

ACTIVATOR PROTEIN-1 (AP-1): A key DNA binding protein that is required to activate the expression of many inflammatory and immune genes.

ACUTE REJECTION: Graft dysfunction of solid organs in the first post-transplant period, reflected histologically by infiltration of mononuclear cells and destruction of graft components by invading effector T lymphocytes (including CTL): In the histological definition there is no particular time period for the occurrence after transplantation.

ACUTE-PHASE REACTION: Increase in the circulating levels of certain serum proteins during infection, trauma or inflammatory reactions.

ADALIMUMAB: Fully human therapeutic monoclonal antibody directed against human TNF-α.

ADAPTIVE (ACQUIRED) IMMUNITY: Antigen-specific, lymphocyte-mediated defense mechanisms that take several days to become protective and are designed to remove the specific antigen.

ADHERENCE: Direct, ligand-mediated cell-cell contact allowing cells to interact.

ADHESION MOLECULE: A protein that enables cells to interact with each other or with the extracellular matrix.

ADJUVANT: A non-immunogenic material, co-administered with antigen that enhances the immune response to the antigen.

ADOPTIVE IMMUNOTHERAPY: Also known as passive immunotherapy. Therapeutic intervention consists of the administration of antibodies or immune cells (typically T cells with predefined specificities, or NK lymphocytes).

ADRENOCORTICOTROPIN: A hormone primarily secreted by the anterior lobe of the pituitary gland which is essential to the growth, development, and function of the adrenal cortex. It is also produced by cells of the immune system. It is often produced in response to stress and is also called corticotrophin.

AFFINITY: The strength with which an antibody molecule binds an epitope (antigenic determinant).

AFFINITY MATURATION: The affinity of a particular antibody is increased as a result of somatic hypermutation and selection of the B cell receptor by competition for pathogens.

AGAMMAGLOBULINEMIA: An immune disorder characterized by very low levels of protective immunoglobulins; affected people develop repeated infections.

AIRWAYS REMODELLING: Changes that occur in asthmatic airways, due to chronic inflammation, leading to stiffening of the airway and altered physiological responses.

ALKYLAMIDES: Biologically active, immunomodulatory compounds, consisting of an alkyl group attached to the nitrogen of an amide, present particularly in the roots

of the purple coneflower (*Echinacea*). Recently, alkylamides have been defined as a new class of cannabinomimetics.

ALKYLATING AGENT: A drug capable of interacting with macromolecules, mainly DNA, to form stable adducts.

ALLERGEN: A substance that induces an allergic immune response, typically involving IgE antibodies.

ALLERGY: Hypersensitivity caused by exposure to an exogenous antigen (allergen) resulting in a marked increase in reactivity and responsiveness to that antigen on subsequent exposure, resulting in adverse health effects.

ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION: Therapeutic intervention by which the recipient's hematopoietic system is replaced by that of a donor who typically is matched for class I and II human leukocyte antigens. The therapy is aimed at eradication of cancer cells when given in the setting of (hematological) malignancies. Cytoreductive pretreatment is given to suppress the recipient's immune response against the donor cells and to reduce tumor cell burden.

ALLOGENEIC TRANSPLANT: A transplant involving organs from individuals of the same species that share different antigens.

ALLOGRAFT, ALLOTRANSPLANT: A transplant involving cells, tissues or organs from individuals of the same species

ALTERNATIVE PATHWAY OF COMPLEMENT ACTIVATION: Pathway initiated by factor B activation at the surface of certain micro-organisms, involving complement factors C3, B, D, P, H and I, leading to the generation of an alternative pathway C3 convertase.

AMBIENT ANALYTE CONCENTRATION: The assay condition in which the ratio of total number of spotted ligands and the target concentration in the analyte are such that the latter is not the limiting factor. As a result the system is independent of sample volume and acts as a concentration sensor.

AMPLIFICATION: *see* T7 amplification

ANABOLIC BIOTRANSFORMATION: Metabolic pathway leading to active metabolites.

ANAKINRA: Therapeutic recombinant human interleukin-1 receptor antagonist.

ANALYTE: The compound to be analyzed in the assay.

ANALYTE SOLUTION: Sample that is analyzed on a microarray for the presence of a particular target.

ANAPHYLATOXINS: Complement peptides (C3a, C4a, C5a) that cause histamine release from mast cells and basophils and smooth muscle contraction, leading to vasodilation.

ANAPHYLAXIS: A type of immune-mediated hypersensitivity reaction. Anaphylactic (type I) or immediate hypersensitivity reactions involve specific IgE antibodies.

ANGIOSTASIS: Cessation of angiogenesis.

ANTHROPOSOPHY: A philosophical school founded by Rudolf Steiner.

ANTIBACTERIAL AGENTS: Ansamycins; benzylpyrimidines; β-lactams; chloramphenicol; cyclines; fosfomycin; fusidic acid; gyrase B inhibitors; isoniazid; lincosamides; macrolides; peptides; quinolones; riminophenazines; sulfones/sulfonamides; other antibiotics (ethambutol, nitrofurans and minimally substituted imidazoles (metronidazole and tinidazole).

ANTIBIOTICS: Therapeutic antimicrobial agents.

ANTIBODY (AB): Immunoglobulin molecule produced by B lymphocytes in response to immunization/sensitization with a specific antigen that specifically reacts with that antigen.

ANTIGEN (AG): Substance to which a specific immunological reaction mediated by either antibody or lymphocyte is directed.

ANTIGEN-PRESENTING CELL (APC): A cell that presents antigen to lymphocytes, enabling its specific recognition by receptors on the cell surface. In a more restricted way, this term is used to describe MHC class II-positive (accessory) cells that are able to present (processed) antigenic peptides complexed with MHC class II molecules to T helper-inducer lymphocytes. These cells include macrophage populations (in particular Langerhans cells, and dendritic or interdigitating cells), B lymphocytes, activated T lymphocytes, certain epithelial and endothelial cells (after MHC class II antigen induction by, e.g., interferon-γ).

ANTIHISTAMINE: Drug that specifically antagonizes receptors for the allergic mediator histamine; used most frequently for H1-receptor antagonists with anti-allergic activities.

ANTI-IGE: Antibody directed against immunoglobulin E; omalizumab is the first therapeutically used monoclonal anti-IgE antibody for the treatment of allergy and asthma.

ANTIMALARIALS: Group of heterogeneous compounds with inhibitory actions on *Plasmodium* parasites, used in the treatment of malaria.

ANTIMETABOLITES: Substances (e.g., some cytotoxic drugs) that replace or inhibit the utilization of endogenous metabolites.

ANTIMICROBIAL PEPTIDE: Host defense peptide of the innate

immune response with a broad spectrum for killing micro-organisms. Examples: Defensins, cathelicidins, LL37.

ANTINOCICEPTION: A reduction in pain sensitivity produced within neurons when endorphin or other substances combines with its receptor.

AORTA-GONAD-MESONEPHROS: Early-stage embryonic structure that gives rise to large blood vessels, gonads and kidney.

APOPTOSIS (PROGRAMMED CELL DEATH): A genetically determined process whereby the cell self-destructs after activation, by Ca^{2+}-dependent endonuclease-induced nuclear DNA (chromosomal) fragmentation into sections of about 200 base pairs. It can be spontaneous (as in neutrophils, explaining the short life span of these cells) or induced (as in tumor cells by interaction with cytotoxic lymphocytes or NK cells).

ARTHUS REACTION: Local immune-complex mediated hypersensitivity reaction, which manifests as local vasculitis due to deposition of immune complexes in dermal blood vessels.

ASTHMA: Respiratory disease due to airway constriction, associated with chronic inflammation and airway remodeling; either due to repeated inhaled exposure to allergen or to other environmental agent.

ASTHMATIC RESPONSE: Constriction of the bronchial tree due to an allergic reaction to inhaled antigen.

ATTENUATED VACCINE: Vaccine based on live bacteria or viruses that are made non-virulent, usually via serial passage *in vitro*. The mechanism by which mutations are introduced via this empirical approach is not well understood.

AUTOANTIBODIES: Immunoglobulins (antibodies) that are directed against endogenous molecules of the host (*see* Autoantigens). They circulate in the serum but may be also detectable in other body fluids or bound in target tissue structures. Autoantibodies may occur as a part of the natural immunoglobulin repertoire (natural antibodies) or are induced by different mechanisms (non-natural or pathological autoantibodies). A number of non-natural autoantibodies are diagnostic markers of defined autoimmune diseases regardless of their pathogenetic activity. They may be directed against conserved non-organ-specific autoantigens, organ-specific autoantigens or cell-specific autoantigens.

AUTOANTIGENS: Self constituents (antigens) of the organism, which may be targets of autoimmune responses mediated by autoreactive B cells (*see* Autoantibodies) or T cells; they include proteins, glycoproteins, nucleic acids, phospholipids and glycosphingolipids.

AUTOFLUORESCENCE: The ability of cells which are NOT labeled with a fluorescent dye to emit a light of a certain wavelength during flow cytometry or fluorescence microscopy. Cells contain molecules which become fluorescent when excited by radiation of suitable wavelength. This fluorescence emission, arising from endogenous fluorophores, is an intrinsic property of cells and is called autofluorescence to be distinguished from fluorescent signals obtained by adding exogenous markers.

AUTOIMMUNE DISEASE: Disease caused by antibodies or T cells targeting self-antigens.

AUTOIMMUNITY: A state of immune reactivity towards self constituents (*see* Autoantigens) that may be either destructive or non-destructive. Destructive autoimmunity is associated with the development of autoimmune diseases.

AZATHIOPRINE: A cytotoxic drug used to treat autoimmune diseases.

B LYMPHOCYTE/CELL: Lymphocytes expressing immunoglobulin (antibody) surface receptors (on virgin B cells IgM and IgD) that recognize nominal antigen and after activation, proliferate and differentiate into antibody-producing plasma cells. During a T cell-dependent process there is an immunoglobulin class switch (IgM into IgG, IgA, IgD, or IgE) with maintenance of the antigen-combining structure. For T cell-independent antigens, cells differentiate only to IgM-producing plasma cells.

BACTERIAL EXTRACT: Aqueous extract of bacteria used as immunostimulant.

BASELINE IMMUNOSUPPRESSION: The standard immunosuppressive regimen given to transplant recipients.

BASILIXIMAB: Therapeutic humanized monoclonal antibody against the interleukin-2 receptor.

BASOPHIL: White blood cells with granules that stain with basic dyes, and which have a function similar to mast cells (*see* Mast cell).

BELL-SHAPED: Symmetrical curve of a normal distribution of data, in reference to immune augmentation.

BIDIRECTIONAL COMMUNICATION: Interactions between the immune system and the brain through a common biochemical language of shared ligands and receptors.

BIOACTIVITY: The effect of a given drug or biological, such as a vaccine or cytokine, on a living organism or tissue.

BIOAVAILABILITY: The degree to which an agent, such as a

drug, becomes available to the target tissue after administration, depending on the degree of absorption of the agent into the circulating blood.

Biological response modifiers (BRMS): Natural, synthetic or engineered products that are used to boost, suppress, direct, or restore the body's ability to fight the disease.

Biologicals: Proteins, poly- or monoclonal antibodies and fusion proteins generated by recombinant DNA technology.

Biopharmaceuticals: Complex macromolecules created by the genetic manipulation of living organisms using biotechnological [gene cloning, recombinant DNA (gene splicing), or cell fusion] technologies rather than chemical manufacturing processes.

Bioterrorism: Hostile release into the environment of human pathological micro-organisms with the intent to cause disease and death.

Biotherapeutic: Therapeutic micro-organism that has a beneficial effect because of its antagonistic activities against specific pathogens for the prevention and treatment of diseases.

Blood dyscrasias: A general term to describe any abnormality in the blood or bone marrow's cellular components, such as low white blood cell count, low red blood cell count or low platelet count.

Bone marrow: Soft tissue in hollow bones, containing hematopoietic stem cells and precursor cells of all blood cell subpopulations (primary lymphoid organ). This is a major site of plasma cell and antibody production (secondary lymphoid organ).

Bradykinin: Locally acting, peptide inflammatory mediator, formed in the blood, with smooth muscle contracting, plasma exudation and pain receptor stimulating properties.

C1–C9: Components of the complement classical and lytic pathway responsible for mediating inflammatory reactions, opsonization of particles and cell lysis.

C2 monitoring: Measurement of drug concentration 2 hours after administration.

Calcineurin inhibitor: Immunosuppressant inhibiting the serine/threonine phosphatase calcineurin which plays a pivotal role in Ca-dependent intracellular signaling and results in blockade of interleukin-2 synthesis involved in T lymphocyte activation.

Cancer immunity: The complex of immune responses (cellular and humoral) that is elicited by cancer cells.

Cancer vaccination: Specific active immunotherapy of cancer (as opposed to adoptive or passive immunotherapy). This intervention consists of the immunization of patients against the antigens that are expressed in cancer cells with the goal of eradicating these cancer cells.

Capsule = virulence factor: Protective structure outside the cell wall of a pathogen that can enable the pathogen directly or indirectly to cause disease.

Capture molecules (ligands): Molecules immobilized on an array for high-affinity and high-specificity binding of the target from a sample.

Carrier: An immunogenic macromolecule (usually protein) to which a hapten is attached, allowing this hapten to be immunogenic.

Caspases: A group of cysteine activated proteases (enzymes) involved in proteolytic degradation during apoptosis (programmed cell death), necrosis and inflammation.

Catecholamine: Vasoactive substance (e.g., adrenaline, noradrenaline, dopamine) synthesized by neurons of the sympathetic and central nervous system and by the adrenal gland medulla.

Cause-for-concern approach: Strategy to decide the need of testing based on adverse effect found in toxicity endpoints, intended use or pharmacological activity of compounds:

CD: Cluster of differentiation, e.g., CD4, CD8, etc. This is a standard naming system for cell-surface proteins of the immune system. For example, CD4 and CD8 identify different subsets of T cells, and CD69 is a cell-surface protein induced upon short-term activation of T cells.

CD3: Molecule consisting of at least four invariant polypeptide chains, present on the surface of T lymphocytes associated with the T cell receptor, and thought to mediate transmembrane signaling (tyrosine phosphorylation) after antigen binding.

CD4: Glycoprotein of 55 kDa on the surface of T lymphocytes and a proportion of monocytes/macrophages. On mature T cells, the presence is restricted to T helper (inducer) cells; the molecule has an accessory function to antigen binding by the T cell receptor, by binding to a non-polymorphic determinant of the MHC class II molecule.

$CD4^+$: T helper cells recognize antigenic peptide in association with MHC class II molecules. They mediate their effector functions by enhancing the persistence of antigen-stimulated T cells or through secretion of effector cytokines.

CD8: Complex of dimers or higher multimers of 32–34 kDa glycosylated polypeptides linked together by disulfide bridges, on the surface of T lymphocytes. On mature T cells, the presence of CD8 is restricted to T cytotoxic-suppressor cells; the molecule has an accessory function to antigen binding by the T cell receptor, by binding to a non-polymorphic determinant of the MHC class I molecule.

$CD8^+$: Cytotoxic T cells recognizing antigenic peptide in association with MHC class I molecules. $CD8^+$ T cells mediate their effector functions by killing the cells presenting the relevant antigenic peptide, or secreting effector cytokines.

CD MARKERS: Cell surface molecules of leukocytes and platelets that are distinguishable with monoclonal antibodies and are used to differentiate cell populations.

CDNA ARRAYS: Microscope slide spotted with several hundreds or thousands of different chemically synthesized forms of DNA called complementary DNA (CDNA), which contains the coding part of gene sequences of interest, complementary to their corresponding messenger RNA (mRNA) transcripts. The immobilized DNA samples on the microarrays are hybridized. The main function of a microarray is to detect the level of mRNA transcript of genes of interest.

CELL ADHESION MOLECULES (CAMS): Group of proteins of the immunoglobulin supergene family involved in intercellular adhesion, including ICAM-1, ICAM-2, VCAM-1 and PECAM-1.

CELL-MEDIATED IMMUNITY: Immunological reactivity mediated by T lymphocytes.

CELLULAR THERAPY: Therapeutic measure involving the isolation of a patient's or control mononuclear cells, *in vitro* manipulation of these cells, and infusion of the modified cells back into the patient.

CENTRAL NERVOUS SYSTEM: The part of the nervous system that includes the brain and spinal cord.

CHEMOKINES: Small-molecular-weight, pro-inflammatory peptide cytokines which attract cells of the immune system (chemotaxis) along a concentration gradient and activate them.

CHEMOTAXINS: Small molecules capable of inducing directed cell movement.

CHEMOTAXIS: Process of directed cell movement, usually to a site of infection in response to a gradient of chemotaxins.

CHIMERIC ANTIBODY: Antibody in which the constant parts of immunoglobulin heavy and light chains of the original antibody molecule are replaced by immunoglobulin sequences of another species.

CHIMERIC PROTEIN: A human-engineered protein that is encoded by a nucleotide sequence made by a splicing together of two or more complete or partial genes.

CHRONIC REJECTION: Graft dysfunction of solid organs late after transplantation, reflected histologically by changes in the vasculature, including remodeling of arteries and arterioles with intimal proliferation; there is no particular time period for its occurrence after transplantation.

CICLOSPORIN: The International Nonproprietary Name (INN) for the drug also known as cyclosporine (US Adopted Name, USAN) and cyclosporin (former British Approved Name, BAN) (*see* Cyclosporin A).

CLASS SWITCH: The shift in a B cell or its progeny from the secretion of an immunoglobulin of one isotype or class to an immunoglobulin with the same v regions but a different heavy-chain constant region and, hence, a different isotype.

CLASSICAL PATHWAY OF COMPLEMENT ACTIVATION: Pathway initiated by immune complexes, e.g., microbes covered by antibodies, involving complement components C1, C2 and C4 and generating a classical C3 convertase.

CLEARANCE: The volume of blood or plasma freed of a drug during a specific time interval.

CLINICAL HYPOTHESIS: Theory to be tested during the clinical phase, such as required dose, frequency, duration, and route of administration, usually predetermined based on data from *in vitro*, animal, human safety, and dose-finding studies.

CLONE: A population of immunocompetent cells that emerges from one single precursor cell; within T or B lymphocytes these are cells with a fixed rearrangement of genes coding for T cell receptor or immunoglobulin.

CO-AND POST-TRANSLATIONAL MODIFICATION: Protein modification occurring during or after protein expression, covalent linking of a chemical moiety to residues of a protein, e.g., phosphorylation, nitrosylation, glycosylation.

COLOMBATION: An as yet still poorly understood process, whereby rapid evaporation of solvents during electrospray ionization results in the explosion of a supercharged micro-droplet, thus projecting some of its constituents into the gas phase as charged ions.

COLONY-STIMULATING FACTORS (CSF): Cytokines predominantly inducing the differentiation from bone marrow

stem cells and the activation of non-lymphocytic leukocytes.

COMBINATION THERAPY: Treatment incorporating two or more types of therapy, i.e., surgery, chemotherapy, radiation, hormonal, gene or drug therapy, etc. If two or more therapeutic measures/agents are taken together, the reduced response and increased resistance rates occurring when a single therapy is given over a prolonged period of time can be overcome.

COMBINATION VACCINE: Vaccine containing antigen derived from more than one pathogen. Examples include diphtheria-tetanus, diphtheria-pertussis-tetanus, diphtheria-pertussis-tetanus-polio, measles-mumps-rubella vaccines.

COMPENSATION: The application of a correcting factor to a fluorescence measurement, which deconvolutes the effects of optical spillover from other fluorochromes contributing fluorescence to a given detector.

COMPLEMENT COMPONENTS B, P, D, H, I: Components of the alternative pathway of complement activation.

COMPLEMENT RECEPTORS (CR1–CR4): Set of four cell surface receptors for fragments of complement factor C3. CR3 and CR4 are integrins.

COMPLEMENT REGULATORY PROTEINS: A collection of plasma proteins that are known to inhibit complement activation at various stages. Examples include C1-inhibitor (C1-inh), factor I, factor H and C4bp.

COMPLEMENT SYSTEM: Series of proteolytic enzymes in blood, capable of lysing microbes and enhancing the uptake of microbes by phagocytes.

COMPLEMENTARITY DETERMINING REGIONS: Three regions (CDR1; CDR2 and CDR3) of amino acid sequence in the immunoglobulin variable region that are highly divergent (hypervariable).

CONTACT PRINTING: A method to deposit probes on a microarray by dipping a solid pin in the probe solution and bringing the pin close to the surface of the carrier thereby releasing a drop onto the carrier.

CORTICOSTEROID: Class of steroid hormones from the cortex of the adrenal gland and synthetic drugs with pronounced anti-inflammatory and immunomodulatory actions, and metabolic and cardiovascular effects.

CORTICOSTEROID RECEPTOR (CR): The cytoplasmic protein to which corticosteroids bind to induce their effects.

CORTICOSTEROID SPARING: Therapeutic agent that when given with a corticosteroid drug, produces the same functional response as a higher dose of corticosteroid.

CORTICOTROPIN-RELEASING HORMONE (CRH): A 41-amino acid peptide primarily secreted by the paraventricular nucleus in the hypothalamus but also by cells of the immune system and the placenta. It controls the secretion of adrenocorticotropin from the pituitary gland and is also involved in antinociception.

COSMID: Type of hybrid plasmid (often used as a cloning vector) that contains so-called *cos sequences*, i.e., DNA sequences originally from the lambda phage. Cosmids can be used to build genomic libraries.

COSTIMULATION, COSTIMULATORY SIGNALLING, COSTIMULATORY PATHWAY: Second signals generated to T lymphocytes after encounter with the antigen *via* the CD3-TCR complex, required to initiate T cell activation. The absence of a costimulation signal can result in anergy (immune tolerance).

COSTIMULATORY MOLECULES: Family of cell surface molecules are also required for antigen presentation (e.g., CD28, CD80, and CD86) apart from the primary molecules involved in antigen presentation, which are MHC-antigen and TCR.

CROMONES: Group of drugs, represented by sodium cromoglycate and nedocromil, with moderate anti-inflammatory activity used in the therapy of allergic rhinitis and mild asthma.

CRYPTIC EPITOPE: A hidden or sequestered epitope that is processed and presented as a result of an inflammatory immune responses initiated by an infectious agent, tissue damage or chemicals such as pharmaceuticals.

CYCLIC AMP: Cyclic adenosine monophosphate (cAMP, cyclic AMP or 3'-5'-cyclic adenosine monophosphate) is a second messenger important in many biological processes. cAMP is derived from adenosine triphosphate (ATP) and used for intracellular signal transduction in many different organisms.

CYCLOOXYGENASE (COX): Enzyme catalyzing the oxidative metabolism of arachidonic acid and other polyunsaturated fatty acids to the biologically active prostaglandins and other eicosanoids. COX-1 is a constitutive isoenzyme, COX-2 an isoform induced by inflammatory stimuli and COX-3 is a neuronal isoform, considered to be the target of the analgesic paracetamol.

CYCLOOXYGENASE PATHWAY: A biochemical pathway for the intracellular production of prostaglandins from arachidonic acid.

CYCLOPENTENONE PROSTAGLANDINS: Prostaglandin metabolites that are characterized by the presence of a highly

reactive electrophilic carbon atom in the unsaturated carbonyl group of the cyclopentane ring.

CYCLOSPORIN A: Immunosuppressive drug inhibiting cytokine synthesis by T lymphocytes (*see* Ciclosporin).

CYTOKINES: Proteins secreted by activated immunocompetent cells that act as intercellular mediators regulating cellular differentiation and activation, particularly within the immune system. They are produced by a number of tissue or cell types rather than by specialized glands and generally act locally in a paracrine or autocrine manner often with overlapping or synergistic actions.

CYTOTOXIC DRUGS: Drugs that directly interfere with DNA/RNA synthesis and, as such, affect cell proliferation.

CYTOTOXICITY: Induced cell death, either by binding of peptide mediators to specific death receptors, by insertion of membrane penetrating components (e.g., complement) or by products released by specific lymphocytes (NK cells, cytoxic T cells) or by granulocytes with cell destroying properties.

DACLIZUMAB: Therapeutic humanized monoclonal antibody against the interleukin-2 receptor.

DE NOVO TREATMENT: Treatment with a (new) immunosuppressant starting at the beginning of transplantation.

DEFENSINS: Antibacterial peptides present in airway surface fluid that are produced by pulmonary epithelial cells, macrophages and neutrophils, and form part of the innate immune defense of the lungs.

DEGRANULATION: Fusion of intracellular granules with the plasma membrane or with the phagosomal membrane, leading to release of granule contents into the extracellular space or into the phagosome, respectively.

DELETIONAL TOLERANCE: The process of tolerance induction by which reactive lymphocytes are removed from the repertoire (for instance by apoptosis).

DENDRITIC CELLS: Leukocytes in tissue that show elongations/protrusions of cytoplasm in the parenchyma, representing a specialized type of antigen-presenting cell derived from lymphocytes or monocytes.

DETERMINING REGION: Amino acid sequence in the immunoglobulin variable region that is highly divergent (hypervariable).

DIAPEDESIS: Process of leukocyte movement from the blood through the endothelial blood vessel wall and basement membrane into the tissues.

DIFFERENTIAL DISPLAY (DDS) ANALYSIS: A powerful technique for analyzing differences in gene expression. This can be useful in identifying novel genes and gene functions. Using DDS, the investigator can directly observe the transcription of thousands of genes and monitor changes in their expression. Such changes may result from mutation, differentiation, or introduction of small molecules or cloned genes.

DIRECT LABELING: Incorporation of labeled nucleotides during cDNA synthesis.

DIURNAL VARIATION: Fluctuations that occur regularly within a 24-hour period.

DNA MICROARRAYS (DNA CHIPS): *see* Microarrays

DNA-PROTEIN MODIFYING AND REMODELLING PROTEINS: Proteins that can alter the number or type of side chains of DNA-associated proteins and thereby modify their interaction with DNA, resulting in changes in DNA compaction and subsequent gene transcription.

DORSAL ROOT GANGLION: A nodule on a dorsal root that contains cell bodies of neurons in afferent spinal nerves.

DRUG RESISTANCE: Condition of lack of sensitivity to a drug that develops during its continued administration, resulting from increased metabolism or changes in target cells.

E-SELECTIN: A member of the family of cell-surface adhesion molecules of leukocytes and endothelial cells that bind to sugar moieties on specific glycoproteins with mucin-like features.

EFFECTOR T CELL: T cell which carries out immune functions, i.e., T helper cells and cytotoxic T cells.

EFFICACY: Capacity or effectiveness of a drug to control or cure an illness. Efficacy should be distinguished from activity.

EICOSANOID: Fatty-acid derivatives, primarily derived from arachidonic acid precursors, that have a wide variety of biological activities. They have 20-carbon fatty-acid derivatives (*eicosa* greek meaning 20). Four main classes of eicosanoids exist – the prostaglandins, prostacyclins, thromboxanes and leukotrienes – derived from the activities of cyclooxygenases and lipoxygenases.

ELISA: An immunoassay system that relies on an enzymatic conversion reaction and is used to detect the presence of specific analytes.

ENDOPEPTIDASE: An enzyme that catalyzes the splitting of proteins into smaller peptide fractions and amino acids by a process known as proteolysis.

ENDORPHINS: One of a family of opioid-like polypeptides originally isolated from the brain but also found in many parts of the body.

ENDOTHELIN: A potent vasoconstricting oligopeptide that plays a key role in vascular homeostasis.

ENDOTOXIN: *see* Lipopolysaccharide.

EOSINOPHILS: (Eosinocyte, eosinophilic leukocyte) are granular leukocytes stained by eosin that contain a typically bi-lobed nucleus and large specific granules. The eosinophils reside predominantly in submucosal tissue and normally low in blood. The cells participate in phagocytosis and inflammatory responses.

EPITOPE: The recognition site on an antigenic protein to which either a specific antibody or T cell receptor binds.

EPITOPE SPREADING: Diversification of epitope specificity from the initial focused dominant epitope-specific immune response to subdominant or cryptic epitopes on that protein.

ERYTHROCYTE: Red blood cell, involved in oxygen transport to tissue. Contains a nucleus in distinct avian species like chickens, but does not have a nucleus in mammals.

ETANERCEPT: Therapeutic fusion protein between the extracellular part of the human type 1 TNF-α receptor and human IgG.

EXOTOXIN: A toxin excreted by a micro-organism, including bacteria and fungi. Exotoxins may have membrane-damaging activity, resulting in hemolysis or cytolysis. Other classes bind to a receptor on the cell surface and stimulate intracellular signaling pathways like the superantigens.

FAB REGION: Region of an antibody that contains the antigen-combining site.

FC RECEPTOR: Cell surface receptor on phagocytes and other (mostly immune) cells involved in the recognition of Fc regions. Fc receptor triggering in phagocytes can trigger various effector functions, including phagocytosis, degranulation and intracellular (oxidative) killing.

FC REGION: Region of an antibody responsible for binding to Fc receptors and the C1q component of complement.

FCεRI: High-affinity receptor for immunoglobulin E antibodies present on effector cells such as mast cells and basophils.

FERMENTATION: An enzymatically controlled chemical transformation of an organic compound.

FERMOSERA: Protease-treated animal sera.

FICOLL: A solution of high-molecular-weight carbohydrate that is used as a density gradient, e.g., for the isolation of mononuclear cells (lymphocytes and monocytes) from whole blood; also used for separation of viable from dead lymphocytes.

FILGRASTIM: Therapeutic recombinant human G-CSF with an additional structural methionine.

FIRST LINE OF DEFENCE: Initial cellular and humoral reaction to pathogen.

FLAVONOIDS: Secondary metabolites of the phenylpropanoid class with important functions as antioxidants.

FLOW CYTOMETRY: The analysis of fluorescence and light scattering properties of cells as they pass in suspension through a laser beam.

FLUORESCENCE-LABELED SAMPLES: Test reagents chemically labeled with a fluorescent marker.

FLUOROCHROME: An organic dye or protein that has fluorescent properties and thus can be conjugated to an antibody for use in flow cytometric staining assays.

FOLLICLES: Round to oval structures in lymphoid tissue, where B cells are lodged. Primary follicles only contain small-sized resting B cells: secondary follicles comprise a pale-stained germinal center, containing centrocytes and centroblasts (B lymphocytes in a state of activation/proliferation), macrophages and the stroma consisting of follicular dendritic cells. This germinal center is surrounded by a mantle (corona) with small B lymphocytes.

FOLLICULAR DENDRITIC CELLS: Cells forming the stationary microenvironment of germinal centers of follicles in lymphoid tissue. They are elongated, often binucleated cells with long branches extending between germinal center cells and forming a labyrinth-like structure. The cells are linked together by desmosomes. The cells are of local parenchymal origin, presumably from pericytes surrounding blood vessels. Their main function is presentation of antigen, trapped as immune complex in the labyrinth, to B lymphocytes.

FORWARD SCATTER: The measurement of light deflected by a cell at narrow angles to the laser beam in a flow cytometer. This measurement correlates with cell size.

FREE RADICAL: Hemicals whose molecular or ionic structure includes an unpaired ("free") electron, usually conferring high reactivity; in biological systems, most free radicals contain oxygen and are produced both by normal as well as pathogenetic biological processes.

FULL GENOME ARRAYS: Array presentations of DNA expressing the whole of a species genome on a single DNA array.

GENE EXPRESSION: Transcription of genes from genomic DNA to messenger RNA.

GENE EXPRESSION PROFILE: The snapshot evaluation on DNA microarrays of the distribution of activated or inhibited genes from a selection of genes taken from cells under specific (e.g., inflammatory) conditions.

GENE THERAPY: Treatment correcting a genetic defect by the introduction of a normal gene into a cell.

GERMINAL CENTER: The pale-staining center in follicles of lymphoid tissue, where B lymphocytes are activated by antigen in a T lymphocyte-dependent manner and subsequently proliferate and differentiate, acquiring the morphology of centroblasts, centrocytes, and plasma cells. The germinal center has a specialized microenvironment made up of follicular dendritic cells. Large macrophages are present, the so-called tingible-body macrophages (starry-sky macrophages).

GLUCOCORTICOIDS OR GLUCOCORTICOSTEROIDS: Potent immunosuppressive and anti-inflammatory drugs derived from the physiological steroid cortisol, the predominant mechanism of which is the inhibition of cytokine synthesis.

GLUCOCORTICOID RESPONSE ELEMENT (GRE): Consensus glucocorticoid receptor DNA binding sequence found in the upstream regions of many corticosteroid genes.

GLUTATHIONE PEROXIDASE(S): Selenium- and non-selenium-containing enzymes that convert lipid peroxides to hydroxyl moieties, thereby reducing their biological reactivity.

GOLD: Gold salts are one of the oldest specific antirheumatic therapies. The anti-inflammatory mechanism of these agents is still unclear.

GRAFT-*VERSUS*-TUMOR: Immune response to tumor cells by immune cells present in a donor's transplanted tissue, such as bone marrow or peripheral blood.

GRAM-POSITIVE BACTERIA: Bacteria with a thick outer cell wall consisting of peptidoglycan and teichoic acid that takes up purple crystal violet Gram stain. In contrast to Gram-negative bacteria, the cell walls of Gram-positive bacteria do not contain lipopolysaccharide.

GRANULOCYTES: Granule-containing myeloid cells, comprising neutrophilic granulocytes (neutrophils), eosinophilic granulocytes (eosinophils) and basophilic granulocytes (basophils).

GROWTH FACTOR: A growth factor is a naturally occurring substance capable of stimulating cellular growth, proliferation and cellular differentiation. Usually it is a protein or a steroid hormone. Growth factors are important for regulating a variety of cellular processes. Growth factors typically act as signaling molecules between cells. Examples are cytokines and hormones that bind to specific receptors on the surface of their target cells.

GROWTH HORMONE (GH): Somatotropin. A 191-amino acid polypeptide hormone of the anterior lobe of the pituitary gland and cells of the immune system that is important in regulating growth.

GROWTH HORMONE RELEASING HORMONE (GHRH): Somatoliberin. A 44-amino acid polypeptide, produced in the neuronal bodies of the arcuate nucleus and cells of the immune system capable of stimulating the release of GH.

HAPTEN: A non-immunogenic compound of low relative molecular mass that becomes immunogenic after conjugation with a carrier protein or cell, and in this form induces immune responses. Antibodies, but not T cells, can bind the hapten alone in the absence of carrier.

HEMATOPOIESIS OR HAEMATOPOIESIS: The formation of blood cells: erythrocytes (red blood cells), thrombocytes (platelets), and leukocytes (white blood cells).

HEMATOPOIETIC STEM CELL: The stem cells that give rise to all blood lineages.

CH_{50} AND AP_{50} HEMOLYTIC ASSAYS: Functional assays, using erythrocyte lysis as a read out, for analyzing respectively activity of the entire (CH_{50}) or alternative plus terminal complement activity (AP_{50}) complement system in serum or plasma.

HEPATITIS B VIRUS: DNA virus specifically infecting the liver, resulting in acute or chronic hepatitis.

HEPATITIS C VIRUS: RNA retrovirus specifically infecting the liver, frequently resulting in chronic hepatitis.

HETEROGENEOUS: Commonly used to describe assays that require a step to separate the bound and free fractions.

HIERARCHICAL CLUSTER ANALYSIS: A general approach to group together objects that are similar to one another.

HIGH MOBILITY GROUP BOX-1 (HMGB-1): Also known as high-mobility group protein 1 (HMG-1) is a DNA binding protein facilitating gene transcription. It is released as a late mediator during inflammation.

HIGH-DOSE CHEMOTHERAPY: Type of chemotherapy treatment in which myeloablative doses are given.

HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART): Combination of typically three or four antiviral drugs of different classes that act at different stages of the hiv life-cycle for treatment of patients with aids.

HISTAMINE: Vasoactive amine released from mast cell and basophil granules.

HISTONE DEACETYLASES (HDAC): Enzymes that remove *N*-acetyl groups from amino side chains of the amino acids of histones, the main constituents of chromatin around which DNA winds, and which therefore regulate gene expression.

HIV INFECTION, DESTRUCTION OF T CELLS: Human immunodeficiency virus targets the CD4 molecule on T lymphocytes, leading to their destruction by cytotoxic T cells or by apoptosis.

HIV INFECTION, OPPORTUNISTIC INFECTION: Because of the suppression of the immune system by chronic human immunodeficiency virus infection, patients become susceptible to a variety of so-called opportunistic infections, which "take advantage" of the compromised defenses of the patient.

HIV INFECTION, REDUCED NUMBER OF T CELLS: Human immunodeficiency virus infection causes a gradual depletion of $CD4^+$ T lymphocytes, which at a threshold of around 350 cells/mL ultimately leads to clinical symptoms of acquired immune deficiency syndrome (AIDS).

HIV INFECTION, TREATMENT: Drug treatment of HIV infection involves combination of viral reverse transcriptase inhibitors and inhibitors of viral proteases. A recently introduced drug (enfuvirtide) inhibits fusion of the virus with the host cell membrane.

HLA: *see* Major histocompatibility complex

HOMOGENEOUS ASSAY: Commonly used to describe an assay that does not incorporate a step to remove the bound from the free label. However, some assays that do not require a separation step still use include a solid phase such as a bead. These are sometimes referred to as nonseparation heterogeneous assays. The term homogeneous assays is reserved for assays that take place entirely in the liquid phase.

HUMAN ANTIBODY (AB): Ab produced by a human cell line and not a mouse cell line after immunization.

HUMANIZED ANTIBODY: Therapeutic monoclonal antibody in which the sequence encoding the CDR of the variable part of the human antibody is inserted into the sequence encoding immunoglobulin of another species.

HUMORAL IMMUNITY: Immunological reactivity mediated by antibodies.

HYBRIDIZATION: The process by which complementary ribonucleic acid strands form double helices.

HYPERSENSITIVITY: Abnormally increased, immunologically mediated response to a stimulus. Sometimes used loosely for any increased response or to describe allergy. The reaction can be mimicked by non-immunological mechanisms (e.g., chemical stimulation of mast cell degranulation).

HYPOTHALAMIC-PITUITARY-ADRENAL AXIS: A complex set of direct influences and feedback interactions among the hypothalamus, pituitary, and adrenal glands. The interactions between these organs constitute the axis, which is a major part of the neuroendocrine system that controls reactions to stress as well as other body processes.

HYPOTHALAMIC RELEASING HORMONES: Any of several hormones produced in the hypothalamus and carried by a vein to the anterior pituitary gland where they stimulate the release of anterior pituitary hormones. Each releasing hormone causes the anterior pituitary to secrete a specific hormone.

ICAM-1 AND ICAM-2: Intercellular adhesion molecules on leukocytes and tissue cells that interact with β2-integrins and mediate binding of leukocytes to other cells.

IDIOSYNCRATIC: Refers to adverse effects of drugs for which an unexpected susceptibility of the host is presumed to be involved. The term covers either adverse effects reflecting a genetic predisposition, or those for which the mechanism has not been elucidated.

IDIOTYPE: Antigenic determinant of the region of the variable domain of antibody molecules or T cell receptor forming the paratope; i.e., the site specifically recognizing a given antigenic determinant.

IGF-1, INSULIN-GROWTH FACTOR: A 70-amino acid polypeptide produced by many types of cells with potent mitogenic effects for a wide range of cells. The liver is a major site of production in response to growth hormone.

IMIQUIMOD: Immunomodulator that is an agonist at Toll-like receptor-7 (TLR-7), stimulating release of cytokines from leukocytes; used topically for the treatment of external genital warts caused by human papilloma virus (HPV).

IMMUNE RESPONSE MODIFIER (IRM): Displays immunomodulatory effects.

IMMUNE SYSTEM: A system including all aspects of host defense mechanisms against xenobiotics and pathogens that are encoded in the genes of the host. It includes barrier mechanisms, all organs of immunity, the innate (immediate, non-specific) immune response effectors (proteins, bioactive molecules and cells – mainly phagocytes) and the adaptive (delayed, specific) immune response effectors (T and B lymphocytes and their products). The two responses (specific and non-specific) act synergistically for a fully effective immune response.

IMMUNOALLERGIC: This term is used to describe immune-mediated hypersensitivity reactions.

IMMUNOGLOBULIN: Immunoglobulins (Ig) are synthesized by plasma cells. The basic subunit consists of 2 identical heavy chains (about 500 amino acid residues,

organized into 4 homologous domains; for μ chain in IgM about 600 amino acid residues, organized in 5 homology domains) and 2 identical light chains (about 250 amino acid residues organized into 2 homologous domains), each consisting of a variable domain and 1–3 constant domains (in the μ chain 4 constant domains). The antigen-binding fragment (Fab) consists of variable domains of heavy and light chain (2 per basic subunit). Five classes of immunoglobulins exist, which differ according to heavy chain type (constant domains): IgG (major Ig in blood), IgM (pentamer consisting of 5 basic units),IgA (major Ig in secretions, here present mainly as a dimeric Ig molecule), IgD (major function as receptor on B lymphocytes), and IgE. Effector functions after antigen binding are mediated by constant domains of the heavy chain (Fc part of the molecule) and include complement activation (IgG, IgM), binding to phagocytic cells (IgG), sensitization and antibody-dependent cell-mediated cytotoxicity (IgG), adherence to platelets (IgG), sensitization and degranulation of mast cells and basophils (IgE). IgA lacks these effector functions and acts mainly in immune exclusion (prevention of entry into the body) at secretory surfaces.

Immunomodulation: Immunomodulation is directed towards either enhancement or suppression of host immunological mechanisms, such as phagocytosis and bactericidal activity, cytokine production, lymphocyte proliferation, antibody response, and cellular immunity.

Immunomodulatory drug/immunomodulator: A drug capable of modifying or regulating, by enhancement or suppression, one or more immune functions. Immunostimulatory drugs may enhance host defence by either stimulating the inflammatory response or inhibiting the anti-inflammatory response. Immunodepressive drugs down-modulate the host response through inhibition of inflammatory mechanisms or induction of counter-regulatory, anti-inflammatory mechanisms.

Immunophenotyping: The use of fluorochrome-conjugated antibodies and flow cytometry to identify subsets of leukocytes in a sample such as peripheral blood. Such assays can be used to identify disease states such as leukemia/lymphoma.

Immunoreceptor tyrosine-based activating motif (ITAM): Signaling motif found in various immunoreceptors on leukocytes that upon phosphorylation of the core tyrosine residue is able to recruit and activate cytosolic tyrosine kinases, including Syk and Zap70.

Immunoreceptor tyrosine-based inhibition motifs (ITIM): Signaling motif found in various immunoreceptors on leukocytes that upon phosphorylation of the core tyrosine residue is able to recruit and activate cytosolic tyrosine phosphatases, including SHP-1 and SHP-2.

Immunosuppression: Defects in one or more components of the nonspecific/innate or specific/adaptive immune system, resulting in inability to eliminate or neutralize non-self antigens. Congenital or primary immunodeficiencies are genetic or due to developmental disorders. Acquired or secondary immunodeficiencies develop as a consequence of immunosuppressive compounds, malnutrition, malignancies, radiation or infection. This may result in decreased resistance to infection, the development of certain types of tumors or immune dysregulation and stimulation, thereby promoting allergy or autoimmunity.

Immunotherapy: Treatment of a disease by the artificial stimulation of the body's immune system to induce or suppress an immune response. *See also* Specific immunotherapy.

Immunotoxicant: Drug, chemical or other agent that is toxic to cells or other components of the immune system. One form of immunotoxicity is the direct toxicity of the compound to components of the immune system, which often leads to suppressed function.This may result in decreased resistance to infection, the development of certain types of tumors or immune dysregulation and stimulation, thereby promoting allergy or autoimmunity. Other types or manifestations of immunotoxicity include allergy or autoimmunity in which the compound causes the immune system to respond as if the compound were an antigen or to respond to self-antigens that have been altered by the chemical.

Indirect labeling: Incorporation of nucleotides labeled with a small molecule like biotin or an aliphatic amine during cDNA synthesis and coupling dyes to the modified nucleotides in a second step.

Induction immunosuppression: The immunosuppressive regimen given at the beginning of transplantation.

Inflammasome: Intracellular multiprotein complex that is responsible for the regulation of inflammatory responses primarily via activation of caspases. The complex comprises, among others, NOD-like receptors, PYCARD (ASC), caspase-1, an NALP and sometimes caspase 5 or caspase 11.The inflammasome promotes the maturation of inflammatory cytokines IL-1β and IL-18.

INFLAMMATION: A complex biological and biochemical process involving cells of the immune system and a plethora of biological mediators (particularly cytokines); it may be defined as the normal response of living tissue to mechanical injury, chemical toxins, invasion by micro-organisms, or hypersensitivity reactions. Excessive or chronic inflammation can have disastrous consequences for the host.

INFLAMMATORY CYTOKINES: Cytokines that primarily contribute to inflammatory reactions, including interferon-γ, interleukin-1, tumor necrosis factor, and chemokines.

INFLAMMATORY SYSTEM: A group of physiologically or anatomically related organs and cells that control the reaction of living tissues to injury, infection or irritation.

INFLIXIMAB: Humanized monoclonal antibody (fusion protein between the variable portions of a murine antibody with the constant parts of human IgG) directed against human TNF-α.

INNATE IMMUNE SYSTEM/INNATE (NONSPECIFIC) IMMUNITY: Non-adaptive, non-antigen-specific host defense system against pathogens and injurious stimuli, present at birth and consisting of phagocytes, natural killer cells and the complement system.

INTEGRINS: Family of heterodimeric cell surface molecules sharing in part a β-chain (β1, β2, β3, about 750 amino acids long), each with a different α-chain (about 1100 amino acids long), that mediate cell adhesion and migration by binding to other cell adhesion molecules (CAMS), complement fragments or extracellular matrix. Based on strong structural and functional similarities, integrins form a protein family rather than a superfamily. Examples: Leukocyte function-related antigen LFA-1 (αL/β1, CD11a/CD18; receptor for ICAM-1, ICAM-2 and ICAM-3); Mac-1 (αM/β2, CD11b/CD18; complement C3 receptor CR3); p150,95 (αX/β2, CD11c/CD18); very late antigens (VLA)-1 (α1/β1, CD49a/CD29; laminin, collagen receptor);VLA-2 (α2/β1, CD49b/CD29; laminin, collagen receptor);VLA-3 (α3/β1, CD49c/CD29; laminin, collagen, fibronectin receptor); VLA-4/LPAM-1 (α4/β1, CD49d/CD29; receptor for fibronectin and VCAM-1);VLA5 (α5/β1, CD49e/CD29, fibronectin receptor); VLA-6 (α6/β1, CD49f/CD29; laminin receptor, and αV/β1, CD51/CD29; vitronectin receptor); LPAM-2 (α4/βp, CD49d/..., or α4/β7).

INTERFERONS (IFN): Antiviral and immunoregulatory glycoproteins induced in different cell types by appropriate (mostly viral) stimuli, conferring resistance to infection with homologous or heterologous viruses.

INTERFERON-α2A, α2B: Therapeutic recombinant IFN-α.

INTERFERON-α2A, OR -α2B PEGYLATED: Therapeutic recombinant IFN-α-conjugated with monomethoxy-polyethyleneglycol (PEG), with a prolonged half-life in the circulation.

INTERFERON-β: Human β-interferons from fibroblasts.

INTERFERON-β1A, β1B: Therapeutic recombinant human IFN-β.

INTERFERON-γ: Cytokine produced by T cells. It induces an antiviral state and is cytostatic for tumor cells. It enhances MHC class I and II expression on various cell types, is antagonistic with IL-4 in IgE/IgG1 synthesis, and stimulates IgG2a synthesis. It activates macrophages to become cytolytic, and natural killer and lymphokine-activated killer activity.

INTERLEUKINS: Heterogenous group of immunoregulatory protein cytokines, also including lymphokines, monokines, interferons, acting as communication signals between cells.They generally have a low molecular mass (<80 kDa) and are frequently glycosylated; they regulate immune cell function and inflammation at picomolar concentrations by binding to specific cell surface receptors; they are transiently and locally produced; and act in a paracrine or autocrine manner, with a wide range of overlapping functions.

INTERLEUKIN-1 (IL-1): Multifunctional cytokine produced by several cell types with pro-inflammatory actions.

INTERLEUKIN-2 (IL-2): Cytokine generated by Th1 cells.

INTERLEUKIN-10 (IL-10): An anti-inflammatory factor produced by Th2 cells, some B cells and LPS activated monocytes.

ISOAGGLUTININS: Specific antibodies agglutinating erythrocytes by reaction with blood group-specific epitopes (isoantigens).

ISOELECTRIC FOCUSING: Technique used in electrophoresis that separates molecules on the basis of their different isoelectric points.

ISOELECTRIC POINT: pH at which a molecule in solution will no longer move in an electric field because it no longer has a net electric charge.

ISOPRINOSINE: Complex salt of inosine used as an immunomodulator with stimulating effects on T cell proliferation.

ISOPROSTANES: Non-enzymatic, biologically active oxidation products of arachidonic acid.

ISOTYPE: Antigenic determinant that defines class or subclass of immunoglobulin molecules.

KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS (KIRs): Also called killer cell inhibitory receptors: Class of receptors present on NK cells that bind to HLA molecules and inhibit cytotoxic reactions induced by NK cells in target cells. KIRs also include activating KIR family members, but these generally do not interact strongly with HLA molecules.

LABEL: The moiety (enzyme, fluorochrome, radioisotope, etc.) used in an immunoassay to quantitate the antigen–antibody interaction.

LATE PHASE: A delayed response to an allergic skin or inhalation challenge. Typically this is seen as swelling or bronchoconstriction 3–8 hours after the initial allergic response.

LECTINS: Unique group of proteins that have been found in plants, viruses, micro-organisms and animals, but despite their ubiquity, their function in nature is unclear. Some lectins possess the ability to induce mitosis in cells (including lymphocytes), which normally do not divide.

LECTIN PATHWAY OF COMPLEMENT ACTIVATION: Pathway initiated by mannose-binding lectin that intersects with the classical pathway.

LEFLUNOMIDE: Synthetic inhibitor of dihydro-orotate dehydrogenase and pyrimidine synthesis with inhibitory activity on lymphocyte proliferation, used therapeutically to treat rheumatoid arthritis.

LENOGRASTIM: Therapeutic recombinant human G-CSF.

LENTINAN: Glucan polymer isolated from the shiitake mushroom (*Lentinus edodes*) with nonspecific immunomodulating and tumor-suppressing activity.

LEUKOCYTES: Also called white blood cells, comprising granulocytes (polymorphonuclear neutrophilic granulocytes, eosinophilic granulocytes and basophilic granulocytes), monocytes, and lymphocytes.

LEUKOTRIENES: A family of biologically active compounds derived by enzymatic oxidation from arachidonic acid. They participate in host defense reactions and pathophysiological conditions such as inflammation.

LEVAMISOLE: An oral immunomodulatory drug that was previously used for eliminating intestinal parasites in animals.

LIGAND: Molecule that binds to a receptor.

LIPOPOLYSACCHARIDE (LPS): Product of some Gram-negative bacterial cell walls that binds to specific toll-like receptors, to CD14 and to LPS-binding protein (LBP). It activates B lymphocytes, macrophages and neutrophils. Also referred to as endotoxin.

LIPOXINS: Biologically active products of arachidonic acid, formed through transmetabolism by two different lipoxygenases in different cells, with pro-apoptotic activity.

LIPOXYGENASE: A non-heme iron dioxygenase critical in leukotriene production.

LYMPH NODE: Lymphoid organ made up of an encapsulated, highly organized structure containing lymphocytes. They usually occur in groups where lymphatic vessels converge (such as in the armpit, groin, neck, lung hila and along the aorta). The lymphocytes are organized in lymphoid follicles within the lymph node. These lymphoid follicles consist of a T cell-rich lymphocyte corona and a B cell-rich germinal center. Lymph nodes are the primary location for antigen presentation and subsequent lymphocyte proliferation and differentiation.

LYMPHOCYTES: Cells belonging to the lymphoid lineage of bone marrow-derived hematopoietic cells. A more restricted designation is that of a small resting or recirculating mononuclear cell in blood or lymphoid tissue, that measures about 7–8 μm and has a round nucleus containing densely aggregated chromatin, and little cytoplasm. Lymphocytes play a key role in immune reactions through specific recognition of antigens.

LYMPHOID ORGAN: Organ in which cells of the immune system, mainly lymphocytes, are lodged in an organized microenvironment, either in a resting stage or in a stage of activation/differentiation/proliferation. Lymphoid organs include bone marrow, thymus, lymph nodes, spleen, and mucosal-associated lymphoid tissue. Central (primary) lymphoid organs are those in which T and B lymphocytes develop and mature (bone marrow, thymus); peripheral (secondary) lymphoid organs are those where immunocompetent lymphocytes recognize antigen, and subsequently initiate immunological reactions and produce effector elements of these reactions.

LYSOZYME: Also known as muramidase, is an enzyme that attacks petidoglycan in bacterial cell walls. Lysozyme is abundant in a number of secretions (like saliva, tears and milk) and a constituent of granules in PMNs.

LYTIC PATHWAY: Complement pathway effected by complement fragments C5–C9, leading to lysis (destruction) of target cells (e.g., Cells to which antibody has bound).

MACROPHAGES: Large 12–20 μm mononuclear phagocytic and antigen-presenting cells, present in tissue (histio-

cyte), constituting the mononuclear phagocytic system that includes monocytes, macrophages, dendritic cells (in lymphoid organs), Langerhans cells (in skin) and Kupffer cells (in liver).

Macrospots: Larger area on a microarray with a high total number of spotted ligands, resulting in assay conditions under which the concentration of target in the analyte is the limiting factor.

Maintenance immunosuppression: The immunosuppressive regimen given when the transplant recipient has reached a stable situation after transplantation.

Major histocompatibility complex (MHC): Set of genes that code for tissue compatibility markers. The MHC complex in man is called HLA (human leukocyte antigens), in mice H2 and in rat RT-1. These markers, which are targets in the rejection of an allograft (matched grafted tissue or organ from a different individual) and hence determine the fate of allografts, also play a central role in control of cellular interactions during immunological reactions. Tissue compatibility is coded by class I and class II gene loci. MHC Class I molecules are coded by the A, B, or C gene locus in the HLA complex, the K and D locus in the mouse H2 complex, in association with the β2-microglobulin molecule. These two-chain molecules are present on all nucleated cells. MHC Class II molecules are coded by the D (DR, DP, DQ) gene locus in the HLA complex, the I-A and I-E locus in the mouse H2 complex, and comprise an α and a β chain (intracellularly associated with an "invariant" chain). The two-chain molecules are present on B lymphocytes, activated T lymphocytes, monocytes/macrophages/interdigitating dendritic cells, and some epithelial and endothelial cells (variable, dependent on species and state of activation), which are also called antigen-presenting cells. Genes within or closely linked to the MHC control certain complement components (MHC Class III genes). MHC restriction is the phenomenon whereby immunological reactions can only occur in association with or parallel to recognition of the polymorphic determinant of a given MHC molecule, and not with that of another MHC molecule. This applies to T lymphocytes with an αβ T cell receptor, which recognize antigenic peptides in association with the polymorphic determinant of MHC molecules and a fraction of the T cell population with a γδ T cell receptor.

Mannan-binding lectin: Protein in blood that can bind to mannan residues on certain micro-organisms and initiate complement activation via the lectin pathway.

Mast cell: Tissue (mainly skin and mucosa)-associated cell activated by antigen/allergen bridging of surface-bound IgE antibodies, releasing enzymes and vasoactive mediators, especially histamine.

Mechanism of action: Complex process whereby a drug, which itself is not the triggering component of therapy, manipulates cellular and cytokine elements of the immune system that are the ultimate antitumor effectors.

α-Melanocortin stimulating hormone (α-MSH): Intermedin; a peptide hormone secreted by the intermediate lobe of the pituitary gland; it causes dispersion of melanin with melanophores (chromatophores), resulting in darkening of the skin, presumably by promoting melanin synthesis within melanocytes.

Membrane attack complex: Assembled complex of complement components C5b–C9 of the lytic pathway that is inserted into target cell membranes and causes cell lysis.

Messenger ribonucleic acid: Messenger ribonucleic acid (mRNA) is a used for transferring information from genomic DNA to ribosomes for protein synthesis.

Metalloproteinase: (or metalloproteases) are a group of proteolytic enzymes whose catalytic mechanism involves a metal. Most metalloproteases are zinc dependent, some use cobalt.

Metallothionein: A family of cysteine-rich, low molecular weight proteins (MW 3500–14 000). They bind both physiological (e.g., zinc, copper, selenium) and xenobiotic heavy metals (e.g. cadmium, mercury, silver, arsenic) *via* the thiol group of integral cysteine residues, which represent nearly 30% of their amino acidic residues.

Methotrexate: Antagonist of folic acid used therapeutically as a cytostatic and as an antirheumatic drug.

Methyl inosine monophosphate (MIMP): Immunomodulator that preferentially augments T cell responses.

Microarray: DNA display technology that allows the monitoring of the whole genome (or parts of it) on a single chip so that researchers can have a better simultaneous picture of the interactions among thousands of genes.

Microbiota: Refers to the community of bacteria and other micro-organisms in the gastrintestinal tract, also referred to as resident flora.

Microrna: MicroRNAs (miRNA) are short RNAs, typically 20–22 nucleotides long, regulating translation of proteins

from mRNAs, either by mRNA degradation or by translation inhibition.

Microvascular leakage: Exudation of blood plasma resulting from the opening of gaps between endothelial cells lining the small vessels of the peripheral blood circulation under stimulation by inflammatory mediators.

Minocycline: Tetracycline antibacterial drug with additional anti-inflammatory activity, especially in inhibiting metalloproteinase activity and inflammatory cytokine release. Also used in the therapy of rheumatoid arthritis.

Mitogen: Substance that activates resting cells to transform and proliferate.

Molgramostim: Therapeutic recombinant human GM-CSF.

Monoclonal antibodies: Identical copies of antibody with the same antigen specificity that consist of one heavy chain class and one light chain type. Typically, monoclonal antibodies are produced by a hybridoma, which is a transformed cell line grown *in vivo* or *in vitro* and is a somatic hybrid of two parent cell lines, one of which is a plasma cell originally producing the single antibody.

Monocyte: Large 10–15-μm non-differentiated mononuclear cell, present in the blood and in lymphatics, comprising the circulating component of the mononuclear phagocyte system.

mRNA: Messenger ribonucleic acid responsible during gene transcription for transferring information from DNA to ribosomes for protein synthesis.

mTOR inhibition, mTOR inhibitors: Immunosuppressants that inhibit the mammalian target of rapamycin, and interfere with an intracellular cytokine driven pathway of cell proliferation.

Mucosa: Structural tissue unit that delineates the lumen of an internal organ (such as the gut or airways) and its orifice. It consists of an epithelial layer next to the lumen. Underneath the epithelium, a region of connective tissue is present, usually containing glands, small blood vessels and smooth muscle cells. Cells of the immune system are dispersed throughout the mucosa.

Mucosa-associated lymphoid tissue: Lymphoid tissue in immediate contact with the mucus-secreting mucosal layer in nasal cavity and nasopharynx (nasal-associated lymphoid tissue), airways (bronchus-associated lymphoid tissue), and intestinal tract (gut-associated lymphoid tissue). Serves as the immunological defense at secretory surfaces, to some extent independent of the systemic (internal) response.

Multiple sclerosis: An autoimmune disease that affects the brain and spinal cord (central nervous system). Symptoms vary dependent on the location and severity of each attack. Episodes can last for days, weeks, or months. These episodes alternate with periods of reduced or no symptoms (remissions). Because nerves in any part of the brain or spinal cord may be damaged, patients with multiple sclerosis can have symptoms in many parts of the body

Multiplex: An assay system for the simultaneous communication of two or more messages or results from the same sample.

Murabutide: Synthetic muramyl peptide that selectively stimulates cytokine release from Th1 lymphocytes, enhancing host defense responses to viruses and bacteria.

Muramyl dipeptides: Bacterial-derived peptides that stimulate host defense against bacterial infection by binding to receptors on macrophages, causing them to release cytokines.

Muteins: Cytokine mutants, generated by gene technology, behaving mainly as receptor antagonists.

NADPH oxidase: Enzyme in phagocytes that generates superoxide, from which other bactericidal reactive oxygen species are derived.

Natural: Product that is extracted from and identical to that in natural organisms.

Natural killer (NK) cells: Lymphocyte-like cells of the innate immune system capable of killing virus-infected and tumor-transformed cells in an antigen-independent manner.

Neo-angiogenesis: The growth of new capillaries from pre-existing vessels.

Neo-epitope: A newly formed epitope on an existing antigen, can be a cryptic epitope or a hapten-carrier conjugate.

Neuropeptide: Peptide released from nerves that acts as a neurotransmitter and/or as a mediator of inflammation.

Neutropenia: Condition characterized by abnormally low numbers of neutrophils in the blood. The severity of neutropenia can be classified as: (i) mild neutropenia [neutrophil counts (numbers/μL blood) between 1000 and 1500] — minimal risk of infection, (ii) moderate neutropenia (neutrophil counts between 500 and 1000) — moderate risk of infection, and (iii) severe neutropenia (neutrophil counts lower than 500) — severe risk of infection. Often treated with G-CSF to promote neutrophil formation and mobilization.

NEUTROPHILS: Highly specialized white blood cells characterized by a multi-lobed nucleus (polymorphonuclear) and a granular cytoplasm that is "neutral" to histological staining under the light microscope. Specialized constituents of the neutrophil membrane, cytoplasmic granules, and cytosol together mediate ingestion and killing of bacteria; after attachment and internalization of the microorganism into the phagocytic vacuole (phagosome), its destruction is mediated by the release of an array of antimicrobial polypeptides and reactive oxidant species.

NITRIC OXIDE: Gaseous local mediator with vasoactive, proinflammatory, bacteriocidal and neurotransmitter activities.

NITRIC OXIDE SYNTHASE: Enzyme catalyzing the synthesis of nitric oxide (NO) from arginine.Occurs in a neuronal form (nNOS, NOS-1), endothelial cell (eNOS, NOS-3) and inducible (iNOS, NOS-2) form.

NOD-LIKE RECEPTORS (NLR): Family of leucine-rich repeat-containing cytosolic proteins that form inflammasomes.

NONADRENERGIC NON-CHOLINERGIC PATHWAY: Interaction between mucosal lymphoid tissue and the peripheral nervous system *via* nonadrenergic non-cholinergic chemical neurotransmitters.

NON-CONTACT PRINTING: A method to deposit probes on a microarray using ink-jet technology avoiding direct contact between the printing needle and the microarray surface.

NON-SPECIFIC IMMUNITY: *see* Innate immunity

NON-SPECIFIC IMMUNOSTIMULATION: Antigen-independent enhancement of the sensitivity to activation by a variety of antigens.

NORMALIZATION: Normalization of microarrays refers to adjustment of microarray data for effects arising from variation in the technology, like different fluorescent dyes or variations during microarray hybridization.

NUCLEAR FACTOR-κB (NF-κB): Ubiquitous, inducible, transcription factor that binds to enhancer elements in most immune and inflammatory cells to stimulate inflammatory genes.

NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN-CONTAINING PROTEIN (NOD1): An intracellular pattern recognition receptor, belongs to the family of NOD-like receptors (NOD1, NOD2) found in leukocytes.

OLIGONUCLEOTIDE ARRAY: Microscope slide spotted with several hundreds or thousands of different chemically synthesized forms of oligonucleotides (short sections of RNA or DNA with <20 base pairs), complementary to their corresponding messenger RNA (mRNA) or complementary DNA, which they are intended to detect.

OM-174: A soluble immunomodulator derived from *Escherichia coli* triacylated lipid a, used as an adjuvant in vaccination.

OPPORTUNISTIC INFECTION: Infection by micro-organisms that are usually harmless but can become pathogenic when host resistance to disease is impaired.

OPSONIZATION: Covering of pathogenic particles and microorganisms with antibody or complement proteins that enhance uptake of these by phagocytes.

OPTIMAL IMMUNOMODULATORY DOSE: Treatment dose that maximally induces the chosen immunomodulatory action with acceptable toxicity.

OSTEOPOROSIS: Metabolic disorder associated with fractures of the femoral neck, vertebrae, and distal forearm.

PASSIVE IMMUNOTHERAPY: Prophylactic or therapeutic treatment of disease by exogenous administration of antibody.

PATHOGEN-ASSOCIATED MOLECULAR PATTERNS (PAMPS): Protein, lipid or DNA moieties specifically expressed by microbes.

PATHOGENIC MICRO-ORGANISM: Micro-organism that is capable of inducing disease in the host.

PATIENT COMPLIANCE: Voluntary cooperation of the patient in following a prescribed regimen.

PATTERN-RECOGNITION RECEPTOR: Molecule present on the surface of most immunocompetent cells, which recognizes microbe-specific common pathogen-associated molecular patterns and produces an immediate defensive response against the invading microorganism.

PEGYLATION: Process whereby polyethylene glycol (PEG) is attached to a protein in order to extend its circulating half-life and thereby enhance its biological activity.

PENICILLAMINE: Thiol-containing drug with immunomodulating activity and copper-chelating activity; previously used as an antirheumatic drug. Also used to treat Wilson's disease.

PEPTIDOGLYCAN: Polymer of sugars and amino acids that forms a mesh-like layer and major constituent of the cell wall of Gram-positive bacteria. Also present in Gram-negative bacteria, but the layer is substantially thinner.

PERIPHERAL NERVOUS SYSTEM: The section of the nervous system lying outside the brain and spinal cord. The peripheral nervous system is divided into the somatic nervous system and autonomic nervous system and connects the central nervous system to the limbs and organs.

PEROXYNITRITE: Highly reactive, tissue-damaging product of the oxidation of nitric oxide.

PEYER'S PATCH: Lymphoid tissue in the wall of the small intestine, separated from the gut lumen by a domed area and an epithelial layer; forms part of the mucosal-associated lymphoid tissue; main function is initiation of immune reactions towards pathogens entering through the dome epithelium.

PHAGOCYTE: Cell (etymologically "devouring cell") that is characterized by its ability to engulf via receptor-mediated endocytosis relatively large particles or micro-organisms (phagocytosis) into intracellular vacuoles by a process generally requiring actin polymerization. This property is essential for the role of phagocytes in host defense. Two main phagocyte lineages exist: Polymorphonuclear cells (polymorphonuclear neutrophils PMN, eosinophils PME and basophils) and mononucleated cells (monocytes and macrophages), referred to as mononuclear or professional phagocytes.

PHAGOCYTOSIS: Process by which phagocytes bind and engulf material >1 μm (e.g., microbes) in an Fc receptor-dependent manner, with accessory help of complement receptors. Phagocytosis occurs *via* a "zipper" mechanism, whereby the particle, opsonized (coated) with antibody or complement, becomes enclosed by the cell membrane of the phagocyte. The particle is then incorporated into a vacuole (phagosome) where it is degraded by proteases and an NADPH oxidase-mediated oxidative burst with formation of superoxide anion, peroxide anion, and hydroxyl radicals.

PHAGOSOME: Intracellular vacuole containing phagocytosed material.

PHARMACOKINETIC MONITORING: The monitoring of pharmacokinetic parameters of a drug or a drug metabolite in order to avoid drug-related toxicity, to maintain therapeutic concentrations or to adjust the administered doses.

PHOSPHODIESTERASE: Enzyme that breaks down cyclic nucleotides (cAMP, cGMP).

PHYTATES: Phosphorus compounds found primarily in cereal grains, legumes, and nuts. They bind minerals such as iron, calcium, and zinc and interfere with their absorption in the body. They also have beneficial heath effects, help disease prevention, and they can be considered as antioxidant compounds in foods.

PLATELET-ACTIVATING FACTOR (PAF): Molecule derived from phosphatidylcholine; released by immune cells and tissue cells; acts as a chemoattractant for and activator of phagocytes as well as an adhesion molecule.

POLYMERASE CHAIN REACTION (PCR): A method to amplify DNA exponentially. The two strands of double-stranded DNA are separated by denaturation using high temperature. By lowering the temperature, two primers bind in reverse orientation on two sides of the DNA fragment to be amplified. A thermostable polymerase prolongs the primers synthesizing a second strand for each DNA strand. Thereby, two double-stranded DNAs are produced for each original double-stranded DNA. By repeating this process several times, the DNA amount is doubled for each cycle leading to an exponential amplification.

POLYMORPHIC ENZYME: An enzyme that displays variable activity as a result of the presence of several variants of the encoding gene.

POLYMORPHISM: The existence of a gene in several allelic forms in a single species.

POLYMORPHONUCLEAR LEUKOCYTE (POLYMORPHONUCLEAR GRANULOCYTE, PMN): Leukocyte of bone marrow origin, with a lobular nucleus, involved in acute inflammatory reactions. Main subsets are basophilic, eosinophilic and neutrophilic granulocytes (showing different cytoplasmic granule colors under hematological staining). The cells contribute to bacterial killing and (acute) inflammatory reactions after attraction by specific (immune complex-mediated) or nonspecific stimuli (including complement components); after activation, they release granules containing various hydrolytic enzymes.

POMC, PRO-OPIOMELANOCORTIN: The precursor protein for ACTH and endorphins.

POST-TRANSLATIONAL MODIFICATION: *see* Co- and post-translational modification

PREBIOTICS: Non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health.

PRECISE: The precision of an assay defines the reproducibility of multiple assay measurements. However, a precise assay is not necessarily accurate.

PRL, PROLACTIN: A peptide hormone of the anterior lobe of the pituitary gland that stimulates the secretion of milk and possibly during pregnancy, breast growth. Also called lactogenic, mammotropic, or galactopoietic factor or hormone; lactotropin.

PROBIOTICS: Live micro-organisms which, when admin-

istered in adequate amounts, confer a health benefit to the host, used as dietary immunomodulators (e.g., *Lactobacillus*).

PROSTACYCLIN: Eicosanoid-family derived lipid mediator with vasodilatatory, antiproliferative, antithrombotic and antiinflammatory actions.

PROSTAGLANDIN (PG): Biologically active product of the oxidative metabolism of polyunsaturated fatty acids by cyclooxygenase. Several different prostaglandins are formed by specific PG synthetases, including PGE2 and PGD2 from arachidonic acid, which are involved in inflammation.

PROTEOME: The protein complement expressed by a genome.

PROTEOMICS: The scientific study of the proteome.

PSEUDO-ALLERGIC: Non-immune-mediated hypersensitivity reactions that bear clinical similarities to immune-mediated hypersensitivity reactions because of the release of the same mediators. The term "anaphylactoid" is often used to depict pseudo-anaphylactic reactions in which non-immune-mediated hypersensitivity reactions are involved.

PSORIATIC ARTHRITIS: A type of arthritis that is often associated with psoriasis of the skin. The arthritis may be mild and involve only a few joints, particularly those at the end of the fingers or toes. Patients who also have arthritis usually have the skin and nail changes of psoriasis.

PURPLE CONEFLOWER (*ECHINACEA PURPUREA*): Indigenous North American plant, an extract of which is used as a mild immunostimulant.

RADIOIMMUNOASSAY (RIA): A test combining radioisotopes and immunological reagents to detect trace amounts of substances.

REACTIVE OXYGEN SPECIES (ROS): Unstable, highly reactive form of oxygen or oxygen-containing molecule (e.g., H_2O_2, superoxide anion, hydroxyl radical), which can be formed, for example, by phagocyte membrane NADPH oxidase and is involved in bacterial killing, protein degradation and acute inflammatory reactions.

RECEPTOR: Cell surface molecule that binds specifically to certain extracellular molecules.

RECOMBINANT: Protein produced *in vitro* from eukaryotic or prokaryotic cells as a result of alteration of the gene for the protein by mutation, addition, or deletion in the laboratory.

REPERTOIRE: The total spectrum of specific antigen-recognizing capacities (diversity) within the population of T or B lymphocytes.

RESPIRATORY BURST: Increase in oxygen consumption of phagocytes following cell activation by opsonized particles or soluble stimuli; leads to generation of superoxide and other reactive oxygen species.

RHEUMATOID ARTHRITIS: Systemic autoimmune disease mediated by autoantibodies and autoreactive lymphocytes, characterized by loss of bone density and painful inflammatory destruction of joint cartilage and bone, leading to deformation of the joints and limbs.

ROLLING: Initial contact between leukocytes and the vascular endothelial cells that acts to slow down the leukocytes, enabling firm adhesion to blood vessel wall.

SAMPLE MATRIX: This refers to the type of sample (e.g., sample, plasma, urine, cell culture supernatant, etc.) to be analyzed. These samples may contain components (e.g., binding proteins) that could potentially interfere in the antigen–antibody interaction.

SCURVY: Disease characterized by exhaustion and bleeding due to vitamin C deficiency.

SELECTINS: Group of three adhesion molecules on immune cells (L-selectin), platelets (P-selectin) and endothelial/epithelial cells (E-selectin, P-selectin) that bind to carbohydrate residues on molecules of opposing cells.

SELENOPROTEINS: Endogenous proteins with or without known functions into which selenium is incorporated as seleno-cysteine.

SENSITIVITY: The limit of detection of an assay. It is usually calculated by determining the mean and standard deviation from multiple measurements of a sample lacking any analyte. The value on the standard curve corresponding to two or three standard deviations above the mean is taken to be the assay sensitivity.

SENSITIZATION: Induction of specialized immunological memory in an individual by exposure to antigen.

SEROTONIN (5-HYDROXYTRYPTAMINE, 5-HT): Vasoactive amine mediator released by platelets, neurons and mast cells, with pro-inflammatory and smooth muscle contracting and neurotransmitter properties.

SERPINS: Serine protease inhibitors. Family of proteins with specific inhibitory action on serine proteases, located in all extracellular body fluids and intracellularly in many cell types.

SERUM THERAPY: Therapeutic treatment of disease by supply with heterologous (animal) serum containing antibody.

SHOCK: Acute hypotensive response resulting from mas-

sive systemic immunological activation of mast cells, with release of histamine (anaphylactic shock) or from systemic bacterial infection, with stimulation of inflammatory cytokine release by bacterial lipopolysaccharide (LPS; septic shock); the latter is often associated with extensive complement activation and inflammatory necrosis of essential organs (multiple organ failure).

SIDE SCATTER: The measurement of light deflected by a cell at wide angles to the laser beam in a flow cytometer. This measurement correlates with cell granularity.

SOMATIC MUTATION: Small non-inherited changes in genes, resulting in alterations in protein amino acid sequences. In immunoglobulin molecules, such changes result in diversity of the antigen binding site (variable region).

SOMATOSTATIN (SOM): Somatotropin release-inhibiting factor; a tetradecapeptide capable of inhibiting the release of GH by the anterior lobe of the pituitary gland.

SPECIFIC IMMUNITY: Immune responses directed towards specific antigen.

SPECIFIC IMMUNOTHERAPY: Administration of allergen extracts to modify the allergic immune response and thereby alleviate the symptoms and progression of allergic diseases.

SPECIFICITY (OF AN IMMUNOASSAY): Defines whether or not compounds other than the analyte can interact with the binding sites of the antibodies used in the assay. This is determined by performing cross-reactivity studies with a series of structurally related compounds likely to be present in the biological samples under investigation.

STEM CELL: A multipotential self-renewing precursor cell of cells in a distinct lineage (for instance, the hematopoietic cell lineage).

STEM CELL TRANSPLANTATION: Infusion of healthy stem cells into patients who have undergone high-dose chemotherapy for leukemia, immunodeficiency, lymphoma, anemias or metabolic disorders.

SUBSTRATE: The target molecule which is modified during the course of an enzyme catalyzed reaction.

SUBUNIT VACCINE: Vaccine containing (purified) antigen derived from pathogens.

SULFASALAZINE: Sulfonamide antibiotic used as an anti-inflammatory agent in inflammatory bowel disease.

SUPEROXIDE ANION: Reactive, higher energetic form of molecular oxygen generated enzymatically by phagocyte membrane NADPH oxidase.

SURROGATE: Biological marker that is considered likely to predict therapeutic benefit and is sufficiently correlated with the primary endpoint.

SYNTHETIC: Man-made product/drug made from sources that are approximate, but need not be identical to the natural product. Synthetic products can come from natural sources, but are manipulated or manufactured.

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE): Human autoimmune disease, usually mediated by antinuclear antibodies.

T7 AMPLIFICATION: Amplification of RNA using T7 DNA-dependent RNA polymerase. RNA *in vitro* is reverse transcribed into cDNA using primers carrying the T7 promoter. After the second-strand synthesis, the T7 promoter is used by the T7 DNA-dependent RNA polymerase for *in vitro* transcription. The T7 DNA-dependent RNA polymerase repeatedly transcribes the same cDNA thereby amplifying the original RNA.

T CELL DEPLETED: In patients who receive myeloablative (bone marrow replacement) therapy and an allogeneic stem cell transplant, removal of the T cells (T cell depletion) from the administered stem cell product has been used to decrease the incidence and severity of graft-*versus*-host disease (GVHD). However, T cell depletion has also been found to significantly delay immune reconstitution and increase the rate of graft failure and tumor relapse.

T CELL IMMUNITY: Immune responses involving activated T lymphocytes as helper or effector (cytotoxic) cells.

T CELL RECEPTOR (TCR): Heterodimeric molecule on the surface of the T lymphocyte that recognizes antigen. The polypeptide chains have a variable and a constant part, and can be an α, β, γ or δ chain. The $\alpha\beta$ T cell receptor occurs on most T cells and recognizes antigenic peptides in combination with the polymorphic determinant of MHC molecules (self-MHC restricted). The $\gamma\delta$ T cell receptor occurs on a small subpopulation, e.g., in mucosal epithelium, and can recognize antigen in a non-MHC restricted manner. The T cell receptor occurs exclusively in association with the CD3 molecule that mediates transmembrane signaling.

T-DEPENDENT ANTIGEN: Antigen for which antibody formation requires T cells.

T-HELPER (TH) LYMPHOCYTES: Functional subset of T lymphocytes that can help to generate cytotoxic T lymphocytes and cooperates with B lymphocytes in the production of antibody.

TH1 AND TH2 RESPONSE: Immune response mediated by particular types of T lymphocytes. T lymphocytes are sub-divided into T-helper ($CD4^+$) and T-effector/cytotoxic ($CD8^+$) lymphocytes. $CD4^+$ T cells assure the regulation

of immune responses via the release of different patterns of cytokines, primarily IL-2 and interferon-γ that characterize Th1 responses, and IL-4, IL-5 and IL-10 that characterize Th2 responses.

T LYMPHOCYTE/CELL: Thymus-derived lymphocytes that induce, regulate, and effect specific immunological reactions stimulated by antigen, mostly in the form of processed antigen complexed with MHC on an antigen-presenting cell. Most T lymphocytes recognize antigen by a heterodimeric α-β surface receptor molecule associated with CD3 molecule mediating transmembrane signaling. Subsets include helper-inducer (Th) and suppressor-cytotoxic (Tc) cells. Th1 and Th2 subpopulations exist: Th1 cells produce interleukin (IL)-2 and IL-3, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and -β, and granulocyte/macrophage colony-stimulating factor (GM-CSF), and function in induction of delayed-type hypersensitivity, macrophage activation, and IgG2a synthesis. Th2 cells produce IL-3, -4 and -5, TNF-α and GM-CSF, and function in induction of IgG1, IgA and IgE synthesis, and induction of eosinophilic granulocytes. Th17 cells produce IL-17. Cytotoxic T lymphocytes differentiate from precursor to effector cytotoxic cells and subsequently kill target cells.

T LYMPHOCYTE SUBSETS: $CD8^+$ cytotoxic T cells recognize antigenic peptide in association with MHC class I molecules. $CD8^+$ T cells mediate their effector functions by killing the cells presenting the relevant antigenic peptide, or secreting effector cytokines. $CD4^+$ T helper cells recognize antigenic peptide in association with MHC class II molecules. They mediate their effector functions by enhancing the persistence of antigen-stimulated T cells or through secretion of effector cytokines.

T REGULATORY CELLS: T lymphocytes that regulate the activity and function of other T lymphocytes.

TACROLIMUS: Immunosuppressive drug inhibiting transcription factor activation of cytokine synthesis in T lymphocytes.

TARGETS: Molecule of interest in an analyte.

TERMINAL COMPLEMENT COMPLEX: Complex of complement factors C5b–C9 that forms upon terminal pathway activation.

TERMINAL HALF-LIFE: The time taken for the plasma concentration of a drug to fall by one-half during the elimination phase.

TERMINAL PATHWAY: Common terminal part of the complement pathway that leads to the formation of the C5b–C9 terminal complement complex that forms lytic pores in the membranes of microbes or target cells.

THERAPEUTIC INDEX: The ratio of the largest dose producing no toxic symptoms to the smallest dose capable of exerting a therapeutic effect.

THERAPEUTIC STRATEGY: A pharmacologically based approach to the treatment of a disease based on the mechanism of action, pharmacokinetics, and toxicology, generally involving multiple modalities that integrate with clinical pathophysiological considerations.

THROMBOCYTE (PLATELET): Small cytoplasmic fragment in blood that is responsible for coagulation. Its main role is to block damaged vessel walls and prevent hemorrhage, by clumping and aggregation. Thrombocytes contain heparin and serotonin, which contribute after release to the acute vascular response in hypersensitivity reactions, and produce oxygen radicals.

THROMBOXANE: Biologically active product of the oxidative metabolism of arachidonic acid by cyclooxygenase and thromboxane synthetase with platelet activating and vasoconstricting activity.

THYROID-STIMULATING HORMONE (TSH, THYROTROPIN): A hormone of the anterior pituitary gland that stimulates thyroid hormone production.

THYROTROPIN RELEASING HORMONE (TRH): Thyroid-stimulating hormone-releasing factor; a tripeptide hormone from the hypothalamus that stimulates the anterior pituitary to release thyrotropin.

TISSUE ENGINEERING: Development and manipulation of laboratory-grown molecules, cells, tissues, or organs to replace or support the function of defective or injured body parts.

TISSUE RESIDENCY: The amount of time a drug spends in a specific tissue – generally the target tissue of interest.

TOLERANCE: A state of unresponsiveness to antigenic stimulation, due to the absence of responding elements or the loss of capacity of existing elements to mount a reaction. Synonym for anergy.

TOLL-LIKE RECEPTORS: Family of pattern recognition receptors with extracellular leucine-rich repeats that bind pathogen-specific molecules and intracellular domains that resemble that of the IL-1 receptor, leading to activation of nonspecific defense responses.

TOPICAL: Route of administration of a drug directly to the site of disease/inflammation.

TRACER: Used synonymously with label in an immunoassay.

TRANSACTIVATION: Stimulation of transcription by a transcription factor binding to DNA and activating adjacent proteins.

TRANSACTIVATION DOMAIN: Region of a transcription factor that stimulates gene transcription.

TRANSCELLULAR BIOSYNTHESIS: A biosynthetic pathway that is dependent on molecules transferred from one cell to another.

TRANSREPRESSION: Inhibition of transcription by a transcription factor binding to DNA and inhibiting adjacent proteins.

TUMOR-ASSOCIATED ANTIGEN: Antigen that is expressed by tumor cells and elicits specific B or T cell-mediated immune responses, irrespective of whether such immune responses affect tumor growth. Tumor-associated antigens include tissue-specific differentiation antigens, cancer-testis antigens, otherwise normal antigens that are overexpressed, fusion proteins, mutational antigens, virus-encoded antigens and minor histocompatibility antigens.

TUMOR NECROSIS FACTOR (TNF): General mediator of inflammation and septic shock originally described as a tumor-degrading factor induced by bacterial lipopolysaccharide. It comprises two forms, TNF-α and TNF-β, produced by monocytes/macrophages; TNF-β is also produced by T lymphocytes and natural killer cells. Tumor necrosis factor has activity similar to IL-1, and acts synergistically with IL-1. It promotes an anti-viral state and is cytotoxic for tumor cells. It stimulates granulocytes and eosinophils, activates macrophages to IL-1 synthesis, stimulates B cells to proliferate and differentiate, and T cells to proliferate, synthesize IL-2 receptor and IFN-γ. It induces fibroblasts to synthesize prostaglandin and to proliferate, it induces fever and synthesis of acute-phase proteins. It reduces cytochrome p450 synthesis. It activates endothelium, promotes adherence of neutrophilic granulocytes to endothelium, and induces cell adhesion molecules like lymphocyte function-associated antigens LFA-1 and LFA-3, ICAM-1, and ELAM-1. It reduces lipoprotein lipase synthesis by adipocytes, and activates osteoclasts to bone resorption.

VACCINE: A substance or group of substances intended to induce an immune response to a tumor or a micro-organism, thus helping the body recognize and destroy cancer cells or micro-organisms. It is usually a preparation of a weakened or killed pathogen, such as a bacterium or virus, or a fragment of the structure of a pathogen that upon administration stimulates antibody production or cellular immunity against the pathogen.

VACCINE DELIVERY SYSTEM: Colloidal carrier allowing multimeric antigen presentation (i.e., containing more than one antigen per particle), thereby increasing the immunogenicity of the antigen; it may also contain adjuvants.

WASH ASSAY: Shorthand for a flow cytometry assay that requires a washing and centrifugation step, as opposed to "no-wash" or homogeneous assays. Sensitivity is generally increased by washing, while homogeneous assays are more convenient.

XENOBIOTIC: Chemical or substance that is foreign to the biological system.

XENOGRAFT, XENOTRANSPLANT: A transplant involving cells, tissues or organs from individuals of different species.

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