I M M U N O L O G Y

CORE NOTES

MEDICAL IMMUNOLOGY 544

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TABLE OF CONTENTS

CHAPTER 1	INTRODUCTION	3
CHAPTER 2	ANTIGEN/ANTIBODY INTERACTIONS	9
CHAPTER 3	ANTIBODY STRUCTURE I	17
CHAPTER 4	ANTIBODY STRUCTURE II	23
CHAPTER 5	COMPLEMENT	33
CHAPTER 6	ANTIBODY GENETICS, ISOTYPES, ALLOTYPES, IDIOTYPES	45
CHAPTER 7	CELLULAR BASIS OF ANTIBODY DIVERSITY: CLONAL SELECTION	53
CHAPTER 8	GENETIC BASIS OF ANTIBODY DIVERSITY	61
CHAPTER 9	IMMUNOGLOBULIN BIOSYNTHESIS	69
CHAPTER 10	BLOOD GROUPS: ABO AND Rh	77
CHAPTER 11	CELL-MEDIATED IMMUNITY AND MHC	83
CHAPTER 12	CELL INTERACTIONS IN CELL MEDIATED IMMUNITY	91
CHAPTER 13	T-CELL/B-CELL COOPERATION IN HUMORAL IMMUNITY	105
CHAPTER 14	CELL SURFACE MARKERS OF T-CELLS, B-CELLS AND MACROPHAGES	111
CHAPTER 15	CELL INTERACTIONS IN HUMORAL RESPONSES: THE CARRIER EFFECT	117
CHAPTER 16	LYMPHOID TISSUE STRUCTURE	123
CHAPTER 17	ONTOGENY OF THE IMMUNE SYSTEM	129
CHAPTER 18	TOLERANCE	135
CHAPTER 19	AUTOIMMUNITY: BREAKDOWN OF SELF-TOLERANCE	143
CHAPTER 20	IMMUNODEFICIENCY	147
CHAPTER 21	IMMEDIATE HYPERSENSITIVITY: ALLERGY	153
CHAPTER 22	VACCINATION	163
CHAPTER 23	TUMOR IMMUNOLOGY	169
APPENDIX	1. PRECIPITIN CURVE	175
	2. LABELING OF ANTIBODIES	177
	3. OUCHTERLONY ANALYSIS	179
	4. ABSORPTION AND AFFINITY PURIFICATION	181
	5. RADIOIMMUNOASSAY (RIA)	185
	6. EQUILIBRIUM DIALYSIS	189
	7. CROSS-REACTIVITY	195
	8. COMPLEMENT FIXATION ASSAY	197
	9. SOUTHERN BLOTTING	199
	10. GENETICS OF INBREEDING	201
	11. MIXED LYMPHOCYTE REACTION	205
	12. PLAQUE-FORMING CELL ASSAY	207
	13. HYBRIDOMAS	209
GLOSSARY		213
INDEX		225

CHAPTER 1
INTRODUCTION

> Resistance to infectious diseases relies on both *INNATE* and *ADAPTIVE* modes of immunity. While both are effective and significant, a major focus of this course is ADAPTIVE IMMUNITY, that mode of immunity which exhibits SPECIFICITY and MEMORY.
>
> Two systems of *adaptive* immunity protect all vertebrates, namely CELLULAR and HUMORAL immunity. The consequences of both of these classes of immune responses may be harmful as well as beneficial, and are mediated by cells of the highly distributed LYMPHOID SYSTEM.

INNATE *versus* ADAPTIVE IMMUNITY

INNATE IMMUNITY

The body's first line of defense against pathogenic organisms (including bacteria, fungi and viruses) is the physical barrier provided by the skin, by the epithelium and mucus secretions of the alimentary tract and lungs, *etc.* This level of protection, however, is relatively *non-specific*; it distinguishes little, for example, between the bacterial organisms Staphylococcus and Streptococcus, or between the viral agents causing polio and smallpox. A next level of defense is manifested by a variety of cells and serum molecules which may promote ingestion and killing of potentially infectious organisms, cells including *macrophages*, *neutrophils* and *dendritic* cells, and molecules including *complement* and *defensins.* These modes of protection are present in all healthy individuals, and are essentially unchanged following repeated challenges by the offending pathogens - that is to say they do not display *memory*, and are collectively referred to as INNATE IMMUNITY. Mediators of innate immunity contribute to the complex process of development of INFLAMMATION. However, as we will discuss later, the mechanisms of non-specific inflammation overlap and interact extensively with those mediating adaptive immune responses, which will be clearly illustrated in the modes of action of dendritic cells, macrophages and complement.

Although relatively non-specific, innate immunity is highly effective and centrally important to our well-being, as evidenced by the consequences of damage to this system by trauma (*e.g.* wounds which damage epithelium and may get infected, ionizing radiation which can inhibit the inflammatory response) or by disease (*e.g.* emphysema, which causes greatly increased sensitivity to bacterial infection in diseased lungs). In fact, as we will see, loss of effective innate immunity can have as deadly consequences as any loss of adaptive immune function.

ADAPTIVE IMMUNITY

What is classically meant when referring to the "immune system", however, is not the non-specific manifestations of innate immunity, but the complex system of immune reactions known as ADAPTIVE IMMUNITY (including both *humoral* and *cellular immunity*, defined

below), which display the closely related features of SPECIFICITY and MEMORY. This course, and these notes, will attempt to define the nature of, and the molecular basis of both of these features.

The discipline of immunology can be approached from two distinct perspectives:

1) **Historical/Medical outlook: *Resistance to Infectious Disease*.** The adaptive immune system can confer *specific* resistance to many infectious diseases, *e.g.,* smallpox. This example illustrates two key features of immune reactions, namely **specificity** and **memory**. Having recovered from smallpox (or having been vaccinated) makes one resistant ("immune") to being infected with smallpox later - the immune system exhibits **memory**. Resistance to smallpox, however, does *not* make a person resistant to *measles, mumps, diphtheria* or other diseases caused by unrelated organisms - the immune system thus shows **specificity**.

2) **Biological approach: *Recognition of "Self" Versus "Non-self"*.** Quite apart from its importance in resistance to infectious disease, the immune system has been of tremendous interest to biologists interested in the nature and mechanisms of *immunological specificity*, one aspect of which can be regarded as an organism's ability to distinguish *self* from *non-self*. The single-celled amoeba, for instance, ingests food by phagocytosis; how does it distinguish a particle of food from one of its own pseudopods? (Note that the same question may be asked of a macrophage.)

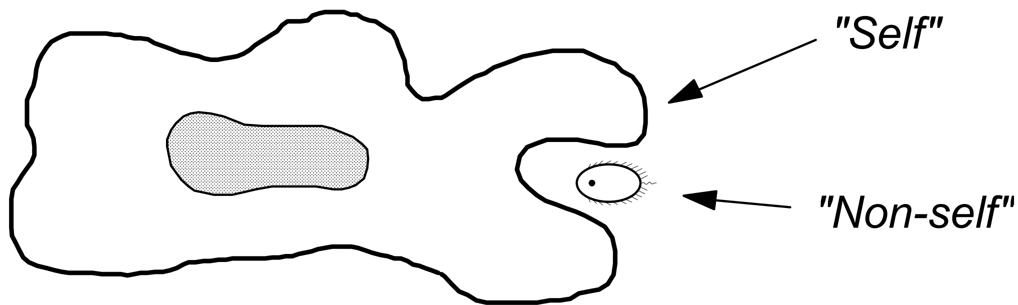


Figure 1-1

For multicellular organisms the problem becomes even more complex -- they must be capable of recognizing their own diversity of normal cell types as *self* while at the same time retaining the ability to recognize *foreign* particles and cells and reacting against them.

The biological concept of *specificity*, particularly in the context of cell surface recognition, extends into many other areas, *e. g.* that of control of cell proliferation and differentiation. As a result, the problem of immunological specificity has attracted study by many scientists whose basic interests lay in the areas of differentiation and tumor biology.

Let's define the following two terms in the context of *adaptive* immunity:

IMMUNITY - ***Acquired resistance to infectious disease displaying specificity at the molecular level***. We've already noted that many factors *other* than the adaptive immune system contribute to resistance to disease, for instance the barrier to microorganisms provided by our skin and other membranes and phagocytic cells ("innate immunity"). These are not *acquired*, however, nor do they exhibit the *specificity* required by this definition, and therefore they are not by themselves considered an expression of the "adaptive immune system".

IMMUNE RESPONSE - ***Reactivity against a target displaying specificity at the molecular level.*** The targets of such reactivity may be disease-producing organisms, or *may be completely harmless substances* such as foreign red blood cells or foreign serum proteins. One major criterion for effective reactivity under *normal* circumstances is that the target be "foreign" to the responding organism, although we shall see that immune responses may be directed against "self" components as well. We shall also learn of many factors that may affect the magnitude of immune responses to various targets.

ROLES OF THE IMMUNE SYSTEM

Resistance to infectious disease. From a medical or evolutionary standpoint, this is highly beneficial, and obviously a central role of the immune system. Deficiency in the ability to mount effective immune responses leads to increased susceptibility to infection by bacteria, fungi and viruses. (The largely discredited idea that the immune system also effectively seeks out and destroys cells which are undergoing neoplastic transformation, *i.e*. "immune surveillance", is discussed in Chapter 23.)

However, the immune system does not always act in a manner beneficial to the organism; *some immune responses result in considerable harmful effects and may be fatal, as illustrated by the following examples*:

Allergy. Immune responses to food and to plant and animal products in our environment may result in the various manifestations of allergies. Hay fever and allergies to foods and animal products are very common, and while they often are not very serious, they may sometimes be life-threatening, such as in the case of severe asthmatic reactions or anaphylactic shock.

Autoimmunity . The normal ability of the immune system to distinguish self and non-self can be disrupted by a variety of influences, resulting in damaging and potentially lethal reactivity to normal "self" components. Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) are two examples of many such autoimmune reactions.

Graft rejection; the rejection of foreign tissues and organ transplants is a "normal" consequence of immunological specificity; however, the ultimate result of immune rejection of a heart or liver transplant, for instance, may be fatal. Much research is stilled aimed at discovering more effective methods to *prevent* immune rejection of grafts, while at the same time maintaining the recipient's ability to resist infectious organisms.

THE LYMPHOID SYSTEM: ORGAN OF IMMUNITY

We will discuss later the many different cell types which are directly or indirectly involved in immune responses. One cell type, however, the LYMPHOCYTE, is centrally involved in *all* adaptive immune responses.

No single, localized organ is responsible for immune reactivity, but rather it involves a wide variety of organs which include **lymph nodes, spleen, Peyer's patches, tonsils, thymus** and **bone marrow**. These are collectively known as the LYMPHOID SYSTEM, by virtue of the fact that they all contain large numbers of the white blood cells known as lymphocytes. Each has a unique structure and role in immune responses, which we will examine in some detail later (Chapter 16).

TWO SYSTEMS OF IMMUNITY PROTECT VERTEBRATES: *HUMORAL* AND *CELL-MEDIATED* ("CELLULAR ") *IMMUNITY*

Immune responses can generally be categorized as either **humoral** or **cellular**.

HUMORAL IMMUNE RESPONSES are those mediated by *antibodies* in various body fluids ("humors"), including blood, saliva and the mucous secretions of the lungs and intestinal tract (the nature and structure of antibodies will be discussed in Chapters 3 and 4, and the cellular basis for humoral immunity in Chapter 15). In general, the humoral response offers protection from infections caused by organisms which are **extracellular**, a category which includes *most bacteria* as well as many of their toxic products (*e.g*. diphtheria and tetanus toxins). Hay fever and food allergies are examples of humoral responses which are harmful to the host.

CELL-MEDIATED IMMUNE RESPONSES are *not* mediated simply by antibodies, but require the direct participation of immunologically reactive cells (to be discussed in Chapters 11 and 12). Cell-mediated immunity is responsible, in general, for resistance to infectious organisms which are primarily ***intracellular***. This includes resistance to *viral infections* as well as to *certain bacteria* (*e.g*., the Mycobacterium responsible for tuberculosis). Immunity to *fungal infections* and *graft rejection* are also largely the responsibility of the cellular immune system.

The ability of immune serum to transfer humoral immunity promoted early studies which identified the relevant effector molecules, the ANTIBODIES. In the next few chapters we will examine the structure and function of antibodies, which are the mediators of *humoral* immune responses, and proceed in later chapters to examine the features of *cell-mediated* immune responses.

CHAPTER 1, STUDY QUESTIONS:

1. What are the defining differences between INNATE and ADAPTIVE immunity?

2. What are the differences between HUMORAL and CELL-MEDIATED immunity?

3. What are some of the biological and medical consequences of immune reactions?

CHAPTER 2
ANTIGEN/ANTIBODY INTERACTIONS

See APPENDIX (1) THE PRECIPITIN CURVE; (2) LABELING OF ANTIBODIES

The defining characteristic of HUMORAL immune responses (which distinguishes them from CELL-MEDIATED responses), is their ability to be *transferred by serum*, and the proteins within serum which are responsible for such immunity are ANTIBODIES. We can formulate intriguingly circular definitions for antibodies and ANTIGENS, and note that the universal property of antibodies is their ability to *specifically bind their cognate antigens*. The *consequences* of such binding, however, can vary considerably, depending on the nature of the particular antigen and antibody involved.

We distinguish the PHYSICAL and the BIOLOGICAL PROPERTIES of antibodies, and the properties of ANTIGENICITY versus IMMUNOGENICITY, and introduce the concept of ADJUVANTS, substances which are capable of increasing *immunogenicity*.

We'll begin by defining three important terms:

ANTIBODY - The molecule present in serum and other body fluids which mediates *humoral immunity*, and which can *bind specifically* to an antigen. Serum which contains antibodies (directed against one or more antigens) is termed an ***antiserum***.

ANTIGEN - A molecule which can be specifically bound by an antibody (typically a protein or carbohydrate recognized as "foreign").

EPITOPE (= "***antigenic determinant***" = "***antigenic specificity***") - The minimum target structure on an antigen which is bound by a particular antibody molecule. A particular antigen molecule may (and generally does) bear *many* different epitopes or "determinants", each of which can be a target for antibody binding.

(NOTE: *Antibodies themselves can serve as antigens*; human antibodies, for instance, are "foreign" to rabbits, and can elicit rabbit antibodies to human antibody molecules. As we will see later, the use of antibodies as antigens has been an extremely powerful tool for understanding antibody structure and genetics.)

DEFINING HUMORAL IMMUNITY

Experimentally defining a *humoral immune response* involves demonstrating that *such immunity can be transferred by serum* (or other fluids). The example below (Fig, 2-1) illustrates some key features of humoral immunity.

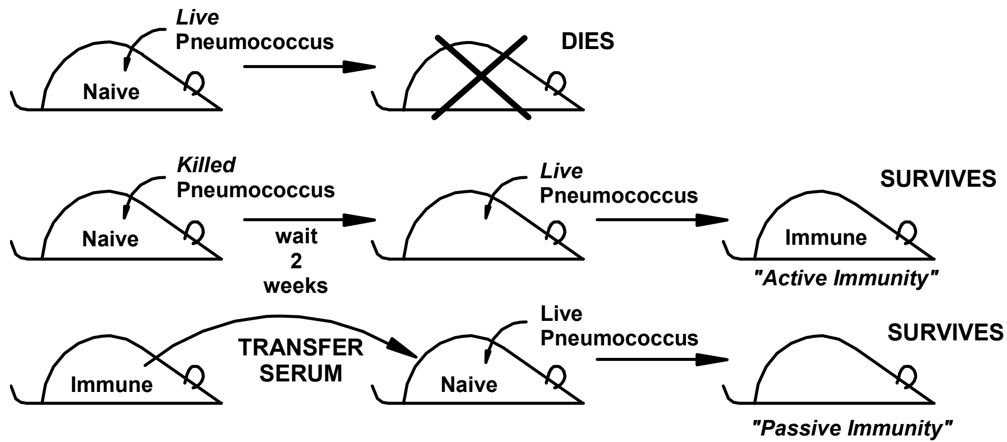


Figure 2-1

If a mouse is injected with a sufficient dose of live Pneumococcus bacteria, it will die of infection within a few days. If, however, it has previously been injected with *killed* organisms, not only does it not succumb to infection, but it will survive a subsequent injection of a normally lethal dose of this organism; such a mouse has been *immunized*, and is therefore said to be *immune* to Pneumococcus. Although not illustrated here, we can further demonstrate that this resistance is specific – the immune mouse will retain normal susceptibility to some other organism to which it had not previously been exposed. Such *specificity* establishes that the immunity we see is a result of the mouse's *adaptive* immune response.

Question: Does this resistance represent *humoral* immunity?

To find out, we take serum from the immune mouse and inject it into a non-immune recipient, then inject a lethal dose of Pneumococcus. We find that this recipient survives this treatment; *serum from an immune mouse transfers immunity* to a naïve recipient. This demonstrates that immunity to this organism is mediated by *humoral immunity*. (NOTE: This does not, however, mean that resistance to *all* bacterial infections is mediated by humoral immunity. As we will see in Chapter 12, transferring serum from a mouse which is immune to another bacterium, Listeria (which is an intracellular pathogen), does *not* confer resistance to naïve recipients; such immunity is therefore *not* humoral.)

This illustration also serves to define two distinct modes of adaptive immunity, namely ACTIVE IMMUNITY and PASSIVE IMMUNITY. Immunization of the mouse in the second line of Fig. 2-1 results in a state of *"active"* immunity; the animal's *own* immune system is responsible for resistance to the subsequent bacterial challenge. On the other hand, transfer of serum, as in line 3 above, results in a state of *"passive"* immunity in the recipient; such immunity is the result of the presence of transferred antibody (see below). The animal's own immune system does not participate at all, and this immunity lasts only as long as sufficient levels of antibody are present.

The substance present in immune serum which is responsible for transferring immunity is **antibody**. In addition to transferring resistance to infection, these serum antibodies can carry out a variety of other functions. For example, if immune serum is mixed with a suspension of Pneumococcus, the bacteria will be seen to rapidly "clump" together. This effect is known as **agglutination**, and is one of the many ways in which antibodies can be detected and quantitated.

The various effects that antibodies may exhibit can be generally categorized as *physical effects*, which depend only on the physical nature of the antibody and antigen, or *biological effects*, which additionally depend on the particular biological properties of the target antigen or other biologically active molecules which are involved.

PHYSICAL EFFECTS OF ANTIBODY

Agglutination. "Clumping" of a *particulate* antigen, *e.g*. bacteria or SRBC (sheep red blood cells). Agglutination of *red blood cells* is a technique which has been widely used in clinical and basic research as well as in the clinical laboratory, and is called HEMAGGLUTINATION. Many soluble antigens can be made effectively particulate by coating them onto SRBC or latex or other particles; the resulting clumping by antibody is known as *passive* agglutination.

Precipitation. Interaction of antibody with a *soluble* antigen to form an *insoluble* complex, *e.g*., with BSA (bovine serum albumin).

In liquid - the precipitate can be recovered by centrifugation and analyzed (see APPENDIX 1, THE PRECIPITIN CURVE). If either the antigen or antibody is radioactively labeled (see APPENDIX 2, LABELLING OF ANTIBODIES), it can be used in a **R**adio**I**mmuno**P**recipitation (RIP) assay, first developed in the 1950s.

In agarose - if the antigen-antibody interaction takes place in a semi-solid medium such as agarose, the resulting precipitate can be easily visualized. This is of special significance in a configuration known as Ouchterlony Analysis (see APPENDIX 3, OUCHTERLONY ANALYSIS).

Precipitation and agglutination are both consequence of *cross-linking* of antigens by antibody into large complexes. The ability of antibodies to carry out this process implies that each antibody can bind at least *two* antigen molecules, and that it can only occur if the antigen molecule has two or more epitopes ("determinants ") which can be recognized by that antibody.

Binding. If an antigen is bound to a solid matrix (latex particles or a plastic dish, for example), and if the antibody is labeled in some way (with a visible, radioactive or enzyme molecule), *binding* of the antibody to its antigen can be easily and sensitively measured. If a radioactive label is used, the assay is called a solid-state **R**adio**I**mmuno**A**ssay (**RIA**). With an enzyme-based label, on the other hand, it becomes an **E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay (**ELISA**). These *solid state assays* (particularly ELISA's) have largely replaced precipitation and agglutination assays in a wide variety of clinical and research applications.

BIOLOGICAL EFFECTS OF ANTIBODY

Protection from infectious disease. We have already seen in the Pneumococcus example (Figure 2-1) how this manifestation of antibody can be assayed by transferring serum from one animal to another.

Immobilization. An antibody directed against components of the flagellae of motile bacteria or protozoa can cause these flagellae to stop moving. This results in the loss of the organisms' ability to move around, and this loss of motility can be detected by microscopic examination.

Cytolysis. If the target antigen is an integral component of the membrane of certain sensitive cells, antibodies may cause disruption of the membrane and death of the cell. This requires the participation of a collection of other serum components collectively known as COMPLEMENT (see Chapter 5), and binding of these components to antibodies is referred to as **"Complement Fixation".**

If the antigen target is a red blood cell, this effect is known as **hemolysis**, which can be readily detected visually. In the case of a bacterial cell target, the effect is referred to as **bacteriolysis**.

If the target is a *nucleated* cell the effect is referred to as **cytotoxicity**, and may be measured by release of a radioactive label incorporated into the cell (such as ^{51}Cr), exclusion of "vital" dyes such as Trypan Blue, or any of several other measures of cell viability.

Opsonization. If the target antigen is *particulate* (*e.g*. a bacterium, or an antigen-coated latex particle), bound antibodies may greatly increase the efficiency with which the particles are phagocytosed by macrophages and other "scavenger" cells. This improvement of phagocytosis is known as *opsonization*, and may be facilitated even further by the presence of complement. As will be discussed later, opsonization is the result of antibodies' increasing the degree to which antigenic particles will "stick" to phagocytic cells. This phenomenon has therefore been referred to as *immune adherence*, and depends on the presence in the membranes of white blood cells of specific receptors either for antibody (**FcR**, or "Fc-receptors") or for complement (**CR**, or "complement receptors"), both of which will be discussed later (see Chapter 14, for example).

ONE COMMON DEFINING PROPERTY OF ANTIBODIES:
ALL ANTIBODIES EXHIBIT SPECIFIC BINDING TO ANTIGEN

Different antibodies may show various combinations of effects; some antibodies may precipitate but not interact with complement (and therefore not show cytolysis), some may be opsonizing but not be capable of agglutination. The single *common feature of all antibodies*, however, is that of *specific recognition and binding to antigen*. All other effects, physical or biological, are secondary consequences of this specific binding. The structure of antibodies and the basis of their ability to specifically bind antigen are the subjects of the next two chapters (Chapters 3 and 4).

ANTIGENS, IMMUNOGENS AND HAPTENS

We have been discussing "antigens" as molecules (1) which can *elicit* antibody production upon injection into an appropriate host; and (2) to which these antibodies can then *bind*. The difference between these two properties is an important one which we will now make explicit by defining two related but distinct terms:

IMMUNOGEN. A molecule which can *elicit* the production of specific antibody upon injection into a suitable host.

ANTIGEN. A molecule which can be specifically recognized and *bound* by an antibody.

These definitions imply that an immunogen must be an antigen, but an antigen is not necessarily an immunogen. Let's illustrate this in the following table:

	Substance	Molecular weight	Immunogen?	Antigen?
1)	BSA	"68,000"	yes	yes
2)	DNP	~200	no	yes
3)	DNP_{10}-BSA	"70,000"	DNP - yes	yes
			BSA - yes	yes
4)	"clarified" BSA	68,000	no (see Note)	yes

If we take a conventional preparation of purified bovine serum albumin (BSA) and inject it into a mouse (line 1 in the table above), the mouse will produce antibodies which will bind to BSA. BSA is therefore *both* an immunogen and an antigen.

If we take the small organic molecule dinitrophenol (DNP) and inject it into a mouse (line 2), *no* antibodies will be produced which can bind DNP. *DNP is therefore not immunogenic*; we will deal with its antigenicity shortly.

We can chemically couple DNP molecules to the protein BSA, yielding DNP-BSA. If we inject this material into a mouse (line 3), we see that antibodies to BSA are elicited (as we would expect), but also find antibodies which will bind specifically to the DNP groups on BSA; we can further demonstrate that these anti-DNP antibodies will also bind *free* DNP (or DNP coupled to any other molecule). Therefore, *DNP-BSA is both immunogenic and antigenic* (with respect to both the DNP groups and the BSA itself), and the free DNP is also *antigenic*, even though we have shown it is *not immunogenic*. DNP is an example of a HAPTEN, a small molecule which is not immunogenic unless it is coupled to a larger immunogenic CARRIER molecule, in this case BSA. (Such a *hapten/carrier* system will be used in Chapter 14 to illustrate the mechanisms of cell interactions required to generate humoral immune responses).

We can further demonstrate that the *immunogenicity of BSA depends on the presence of aggregates of BSA molecules*. If we take a sample of our BSA and centrifuge it at high speed we can remove any aggregated material, leaving behind only single, monomeric BSA molecules in solution. If we immediately inject this "*clarified*" BSA into a mouse we find that it does *not* elicit the production of antibodies (as seen in line 4); this monomeric BSA is

therefore *not* immunogenic (nor can it serve as an effective carrier for a hapten). It is still antigenic, however, which we can show by reacting it with the anti-BSA antibodies which we made against the non-clarified BSA (in line 1, for example).

(NOTE: "Clarified" BSA not only fails to induce antibody formation, but can induce a state of TOLERANCE to BSA, defined as *the specific inability of the mouse to respond to subsequent injections of normally immunogenic BSA*. The mechanism of such tolerance will be discussed in Chapter 18.)

REASONS FOR LACK OF IMMUNOGENICITY

Substances may lack immunogenicity for a variety of reasons:

1) *Molecular weight too low*. Haptens, for example, are not immunogenic until they are coupled to a high molecular weight carrier. There is no simple cutoff for required molecular weight, however; we have already seen that even the 68,000 mw of BSA is not sufficient to be immunogenic unless the molecules are aggregated into even larger complexes.

2) *Not foreign to host*. The adaptive immune system normally responds only to "foreign" substances. A sheep, for instance, will normally *not* make antibodies against its own red blood cells (SRBC), although SRBC are highly immunogenic in mice. (The basis of normal SELF-TOLERANCE is covered in Chapter 18).

3) Some molecules are intrinsically poor immunogens for reasons which are not well understood. Lipids, in general, are poor immunogens, probably because they do not have a structure rigid enough to be stably bound by antibodies. Nucleic acids are also relatively weak immunogens, although they are nevertheless common targets for antibodies present in various autoimmune diseases (discussed in Chapter 19)

HOW TO INCREASE IMMUNOGENICITY: ADJUVANTS

(See also CHAPTER 22)

An ADJUVANT is any substance which, when administered together with an antigen, increases the immune response to that antigen. One of the most widely used adjuvants (in animals but *not* in humans) is FREUND'S ADJUVANT, which consists of mineral oil, an emulsifying agent, and killed Mycobacterium (the organism which causes tuberculosis). A solution of the desired antigen in water or saline is homogenized with this oil mixture, resulting in a **water-in-oil emulsion** which is injected into the recipient. Its powerful adjuvant properties result from several factors:

1) The antigen is *released from the emulsion over an extended period of time*, causing a continuous and more effective stimulation of the immune system. (Antigen given in soluble form will typically be cleared in a matter of hours or days, whereas it can persist for weeks or months in a depot created by the adjuvant.)

2) The Mycobacteria contain substances which *non-specifically stimulate the immune system*, resulting in a higher level of response to the specific antigen. One of these substances which has been extensively studied is Muramyl Dipeptide (MDP).

Although Freund's Adjuvant is not used in humans, other forms of adjuvant can be used, such as *alum precipitation* of antigen, by which a soluble antigen is precipitated together with aluminum hydroxide, resulting in particles of the salt coated with antigen. A soluble antigen is thus converted to a *particulate* form, and again is released from the mixture over an extended period of time. Substances such as purified MDP and others are also being used to develop effective adjuvants which are less toxic, and therefore potentially usable in humans (see Chapter 22)

CHAPTER 2, STUDY QUESTIONS:

1. Define ANTIBODY, ANTIGEN, IMMUNOGEN and HAPTEN.

2. How would you determine if a particular immune response is a HUMORAL response?

3. Describe assays which could be used to measure AGGLUTINATION, PRECIPITATION, HEMOLYSIS and OPSONIZATION.

4. Describe *two* antibody assays which require no antibody function other than specific binding to an antigen.

5. Define and distinguish ACTIVE *versus* PASSIVE immunity.

CHAPTER 3
ANTIBODY STRUCTURE I

See APPENDIX: (3) OUCHTERLONY ANALYSIS; (6), EQUILIBRIUM DIALYSIS; (7) CROSS-REACTIVITY

> Electrophoretic separation of serum proteins identifies the GAMMA-GLOBULIN fraction as containing the majority of antibodies. Three terms which are often confusingly interchanged are defined and distinguished (GAMMA-GLOBULIN, IMMUNOGLOBULIN, ANTIBODY), as are two terms describing antibody/antigen binding, AFFINITY and AVIDITY.
>
> All antibodies are made up of one or more *IgG-like subunits*, each of which has exactly two antigen-combining sites. The *affinity* of these sites for their antigen (defined as the K_{eq} of the binding reaction) is highly heterogeneous in any normal immune response. While the *avidity* of an antibody (its ability to form stable complexes with antigen) does depend on its intrinsic affinity, it also increases dramatically with an increasing number of combining sites per antibody.

In order to determine the structure of antibodies, we first must have a way of isolating these molecules in relatively pure form. We'll begin by describing the general process of serum fractionation, then go on to analyze the nature of antigen-antibody binding.

The many components of normal serum can be separated from one another by various means:

Salt precipitation. Ammonium sulfate [$(NH_4)_2SO_4$] as well as a variety of other salts can be used to precipitate serum components; different proteins will precipitate at different concentrations of salt, providing a convenient means of separating them. The fraction containing most of the antibody activity generally precipitates at relatively low salt, at about 30-40% of saturated ammonium sulfate. This is a very widely used experimental method for fractionation of serum components (and proteins in general).

Ethanol precipitation. Ethanol can also be used to precipitate serum components, which come out of solution at different concentrations and under different conditions of ionic strength, pH and temperature. This is a more elaborate procedure to carry out than salt fractionation, but is the basis for **Cohn Fractionation**, which in modified form remains a standard procedure for preparing serum protein fractions for clinical use more than sixty years after its original description in the 1940's.

Electrophoresis. Different serum proteins migrate at varying rates in an electric field, a property which can be used to separate them. While this procedure can be adapted for use on a preparative scale, it is most commonly used for analysis.

A typical pattern generated by electrophoresis of a serum sample (*e.g*. on a filter paper strip) is shown in Figure 3-1.

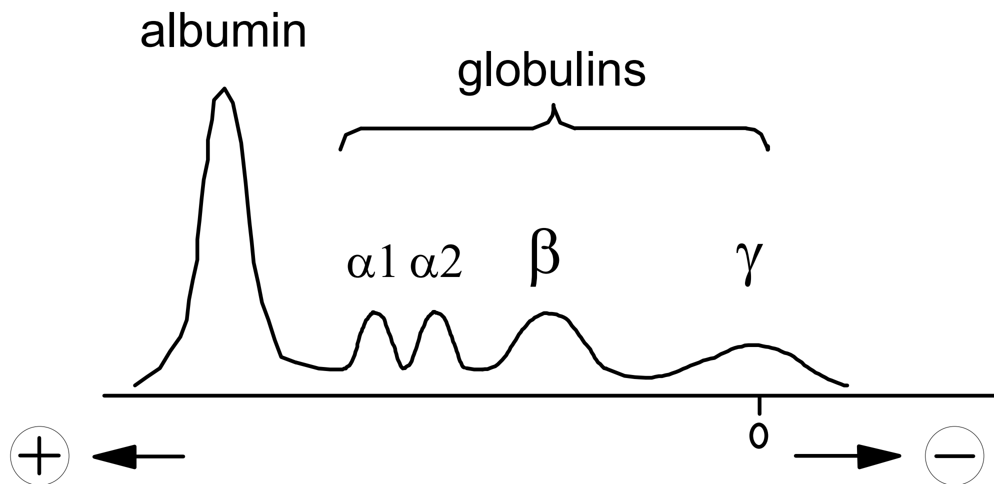


Figure 3-1

Several important points emerge from this pattern:

1) Most serum proteins carry a *negative charge*, and therefore tend to migrate from the point of origin (labeled "O") toward the anode, the positively charged electrode.

2) Four major peaks are seen in this example; these are named (from the anodal, or positive, side) the *albumin* peak (which is by far the largest), followed by four globulin peaks, *α1- and α2-globulin, β-globulin and γ-globulin*.

3) This pattern is *deceptively simple*; serum actually contains *hundreds* of known proteins. Thus, "β-globulin" is not a single protein, but a mixture of *many* components which all happen to migrate in a particular region on electrophoresis.

4) *Most (but not all) antibodies migrate in the γ -globulin region*.

5) The γ-globulin peak is markedly *broader* than the others, reflecting the high degree of *heterogeneity* of the antibodies it contains. This heterogeneity is so great that some antibody molecules in fact migrate in the positions characteristic of α-globulin or β-globulin.

6) The γ-globulin peak is generally centered near the origin, labeled "O"; this reflects the fact that antibodies as a group are relatively neutral, *i.e. less highly charged* than most other serum components.

Three easily confused terms are all commonly used to refer to antibody molecules, ***gamma-globulins, immunoglobulins*** and ***antibodies***. To avoid this confusion let's explicitly define each of them:

GAMMA-GLOBULIN -- Any molecule which migrates in the gamma-globulin peak on electrophoresis. Most, *but not all*, antibodies are in this category, although the term is often used to refer to antibodies in general. (Other serum components migrate in this region as well; therefore, strictly speaking, not all gamma-globulins are antibodies.)

IMMUNOGLOBULIN -- A family of molecules (to which *all* antibodies belong) with similar structures and physical properties. We shall see that these involve homologous amino acid sequences, similar "domain" structures and similar quarternary structures (the ways in which different polypeptide chains are joined into a larger functional unit).

ANTIBODY -- A molecule belonging to the Immunoglobulin family, with binding specificity for a particular antigen. While all antibodies are immunoglobulins, most but *not* all antibodies are gamma-globulins.

Note that our definition of "antibody" requires knowledge of the *binding specificity* of the molecule. If one is dealing with an "antibody" molecule whose specificity is not known, or is irrelevant, it is more accurate to refer to it simply as an "immunoglobulin". (Common usage of these terms varies considerably, however.)

ANALYSIS OF THE ANTIBODY COMBINING SITE: VALENCY, AFFINITY AND AVIDITY

If we immunize a rabbit with DNP-BSA, we can obtain an antiserum which contains antibodies to both the hapten and the carrier protein. This antiserum will precipitate DNP-BSA in addition to DNP-KLH (Keyhole Limpet Hemocyanin, an unrelated protein carrier). If we attach DNP to SRBC (sheep red blood cells) or to latex particles, we can show that the antiserum is capable of showing agglutination (and possibly hemolysis in the case of SRBC).

We can use these antibodies to the DNP hapten in order to learn about antibody structure and function. Specifically, we will ask two questions:

1) How many hapten molecules can a single antibody molecule bind (*i.e* how many combining sites does it have, or what is its “*valency*”)?
2) What is the strength of binding of the hapten to its combining site(s) on the antibody molecule (*i.e.* what is the *affinity* of the combining site)?

We have previously made the prediction that in order for an antibody molecule to be capable of precipitation or agglutination it must have at least two combining sites, in order to permit *cross-linking* of the antigen into large, insoluble complexes. We can determine the *actual* number of combining sites of our anti-DNP antibodies, as well as their affinity, by several techniques; one of them, EQUILIBRIUM DIALYSIS, is discussed more fully in APPENDIX 6, and we will use the results of such an analysis as the basis for our discussion below.

RABBIT IgG ANTIBODIES HAVE TWO HAPTEN-COMBINING SITES

The structure of rabbit IgG antibodies represents the basic structure of all antibodies and we can show by equilibrium dialysis that *each anti-DNP antibody molecule can bind exactly two DNP molecules*. Thus, our minimum prediction of at least two combining sites is fulfilled. Other kinds of antibodies can be shown to have more than two combining sites (IgM and some IgA), but we will see that such antibodies are always made up of multiple units of the basic "IgG-like" structure, each of which bears precisely two combining sites.

CONVENTIONAL ANTIBODIES ARE *HETEROGENEOUS* WITH RESPECT TO AFFINITY

The DNP hapten is bound to each combining site by non-covalent forces, and the strength of this binding is measured by the equilibrium constant of the binding reaction, known as the AFFINITY. The antiserum we describe above contains anti-DNP antibodies with *many different affinities*, typically ranging from 10^5 to 10^{10}. (Antibodies certainly exist with affinities outside this range, but such values are difficult to determine accurately due to technical limitations.)

This *antibody heterogeneity is a hallmark of the immune response*, and has many practical and theoretical implications (see discussions of Clonal Selection and Affinity Maturation [Chapter 7], and Isotype Switching [Chapter 9]). The *broadness* of the gamma-globulin peak on serum electrophoresis (which we have already described) is one consequence of this heterogeneity; in fact, a sharp, narrow gamma-globulin peak (representing a homogeneous protein) is a pathological sign of a **myeloma** or other **monoclonal gammopathy**. However, homogeneous antibodies known as HYBRIDOMAS, or MONOCLONAL ANTIBODIES can be generated experimentally, and are important in many research and clinical applications (see APPENDIX 13).

ANTIBODY AVIDITY: ABILITY TO FORM STABLE COMPLEXES WITH ANTIGEN

AFFINITY is a thermodynamically defined term representing the strength of interaction of a *single* combining site with its hapten. Naturally produced antibodies always have two or more sites, however, so that affinity does not tell the whole story with respect to antigen-binding. A bivalent anti-DNP antibody, for example, can simultaneously bind to *two* DNP haptens on a single BSA molecule, resulting in a much more stable complex than if it only bound to a single site.

AVIDITY, on the other hand, is the term used to describe the ability of an antibody to *form stable complexes* with its antigen. Avidity, of course, depends partly on affinity; all other things being equal (which they rarely are), one IgG antibody with a higher affinity for DNP than another will also have a higher avidity. However, various other factors also play a role, such as the number and spacing of the epitopes on the antigen, the distance between the combining sites on the antibody, and properties such as the "flexibility" of the particular antibody molecule.

Avidity does not have a formal thermodynamic definition, and is most commonly used only in a *relative* context (by demonstrating that one antiserum may exhibit a higher or

lower avidity than another). Nevertheless, in discussing the interaction of an *intact antibody* (which is at least bivalent) with a conventional antigen (which is *almost always* highly multivalent), one must almost always think in terms of "avidity" rather than "affinity". This is of particular importance when considering the biological effectiveness of antibodies which have more than two combining sites, such as serum IgM and some IgA.

CHAPTER 3, STUDY QUESTIONS:

1. Define the terms ANTIBODY, IMMUNOGLOBULIN and GAMMA-GLOBULIN.

2. How is EQUILIBRIUM DIALYSIS carried out, and what can it measure?

3. Define and distinguish antibody AFFINITY and AVIDITY.

CHAPTER 4
ANTIBODY STRUCTURE II

See APPENDIX: (4) AFFINITY CHROMATOGRAPHY; (5) RADIOIMMUNOASSAY

The "IgG-like" subunit of all human antibodies consists of two identical LIGHT CHAINS and two identical HEAVY CHAINS, and proteolytic digestion can be used to establish the different functions of distinct portions of antibody molecules. The nine different CLASSES and subclasses (ISOTYPES) of human immunoglobulins exhibit different physical and biological properties, determined by the amino acid sequence of the CONSTANT REGION of the heavy chains. On the other hand, the VARIABLE REGION of heavy and light chains together form the ANTIGEN-BINDING SITES of antibodies.

The DOMAIN organization of antibodies, and an understanding of the COVALENT and NON-COVALENT interactions involved in their structure and in their interaction with antigen, form the basis for understanding their distinct roles in PRIMARY and SECONDARY IMMUNE RESPONSES, and the key features of IMMUNOLOGICAL MEMORY.

ANTIBODY HEAVY AND LIGHT CHAINS: PROTEOLYTIC FRAGMENTS

In order to examine the structure of antibodies, we will again start with a "typical" IgG molecule, such as the rabbit antibody we discussed at in the last chapter. A schematic diagram of an IgG molecule, together with its proteolytic fragments, is shown below:

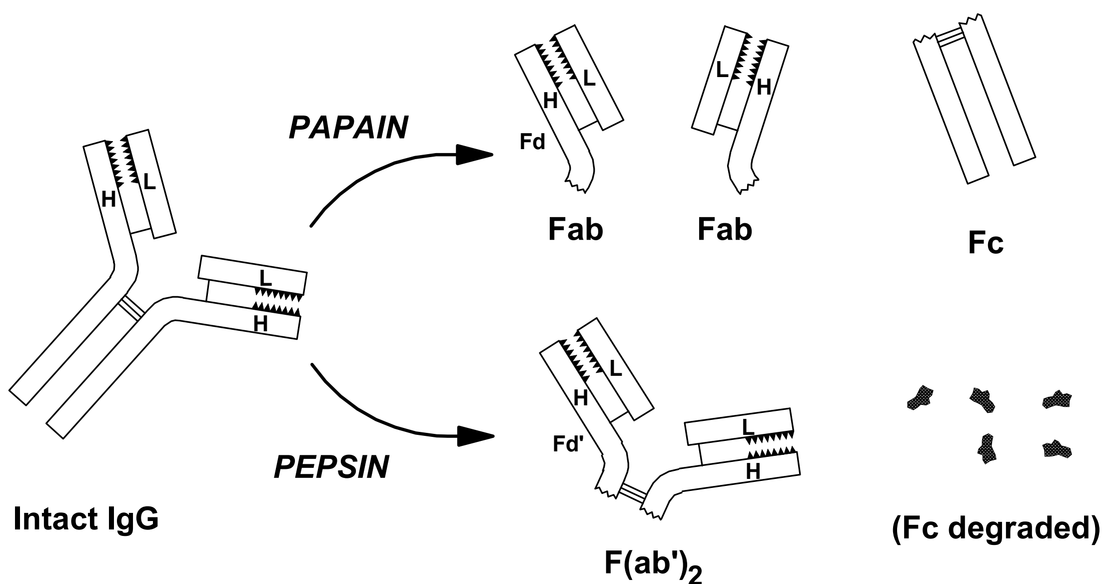


Figure 4-1

- Intact IgG has a molecular weight of ~150,000.
- Each IgG molecule consists of **two identical heavy chains** (*mol.wt.~50,000*) and **two identical light chains** *(mol.wt.~25,000)*. The two heavy chains are linked to each other by one or more disulfide bonds (shown as lines in the diagram), and each light chain is linked to one heavy chain by a single disulfide bond. Immunoglobulin heavy and light chains, as we will see later, are encoded by evolutionarily related members of a large *multigene family*.

This molecule can be broken down into smaller fragments by a wide variety of enzymatic and chemical treatments. Of particular importance has been the use of *two proteolytic enzymes, papain, and pepsin*. Treatment with papain yields two kinds of fragments, a pair of F(ab) fragments, and one Fc fragment. Treatment with pepsin yields a single fragment, the $F(ab')_2$ (the Fc region is degraded by this enzyme).

If we begin with an antibody molecule of known antigen specificity, we can study the fragments generated in this way not only with respect to their physical properties, but also for their ability to interact with antigen and complement. Their properties are as follows:

Fab size ~50,000 Da
binds antigen (**ab** = **a**ntigen-**b**inding)
does *not* show precipitation or agglutination
will *not* fix complement or opsonize

F(ab')$_2$ size ~100,000 Da
binds antigen
can show precipitation and agglutination
will *not* fix complement or opsonize

Fc size ~50,000 Da
cannot bind antigen
is **c**rystallizable
does not fix complement (except very poorly)

[Fd that portion of the *heavy* chain which is included in the Fab fragment**]**

Thus we see that proteolytic cleavage can separate IgG into fragments with *different functional properties*. The F(ab) and $F(ab')_2$ fragments are capable of binding antigen (they contain the antigen binding sites), but only the $F(ab')_2$ can exhibit agglutination or precipitation; this is because these functions require *bivalent binding for cross-linking*. Each F(ab) fragment has *one* antigen-combining site that requires the presence of both the heavy chain and the light chain. In addition, we see that *without the Fc piece, complement fixation does not take place*.

The Fc piece, on the other hand, does *not* bind antigen; and while it is required for complement fixation, it cannot significantly bind complement on its own. The fact that it can be made to form *crystals* was recognized very early as a striking property of the Fc (hence its name). Antibodies in general do *not* form crystals, as a result of their characteristic heterogeneity. The Fc fragment does not share the major source of this *heterogeneity* (variation in the antigen combining site) since it does not contain a binding site.

IMMUNOGLOBULIN CLASS (ISOTYPE) IS DETERMINED BY THE HEAVY CHAIN

So far we have discussed the properties only of a "typical" rabbit IgG molecule. If we look at human immunoglobulins, we see that a variety of different forms exist, and this variety is typical of mammals in general. Five major **classes** of immunoglobulin are known, IgG, IgM, IgA, IgD and IgE; in addition, there are four **subclasses** of IgG, namely IgG1, IgG2, IgG3 and IgG4, and two subclasses of IgA, namely IgA1 and IgA2, yielding a total of nine different classes and subclasses (="*isotypes*"; see Chapter 6) of human immunoglobulin.

PROPERTIES OF HUMAN IMMUNOGLOBULINS

Class	**sub-classes**	**serum conc. *mg/ml***	**sedim. coeff.**	**size**	**# binding sites**	**H-chain class**	**Biological properties**
IgG	**4**	12	7S	150 KDa	**2**	**γ (γ1, γ2, γ3, γ4)**	**C-fixing, placental X-fer**
IgM	-	1	19S	900 KDa	**10**	**μ**	**C-fixing, B-cell surface Ig**
IgA	**2**	2	7,9,11S	(160 KDa)n	**2,4,6**	**α (α1, α2)**	**secretory Ig**
IgD	-	0.03	7S	180 KDa	**2**	**δ**	**B-cell surface Ig**
IgE	-	0.0003	8S	200 KDa	**2**	**ε**	**"reaginic" Ig**

The **class** (and **subclass**) *of an immunoglobulin is determined by the kind of heavy chain it bears*. Nine different heavy chains define the nine classes and subclasses (**isotypes**) of human Ig.

All immunoglobulins share the same pool of light chains; there are two types of light chains, kappa and lambda. Thus, an IgG1 molecule has two gamma-1 heavy chains, and may have. Likewise for an IgM molecule which has *mu* heavy chains, and may have *either* kappa or lambda light chains, but not both...etc.

All immunoglobulins share the same basic "IgG-like" structure consisting of two linked heavy chains and two light chains. IgG, IgD and IgE have precisely this structure, and differences in their molecular masses (seen in the table above) are due to differences in the size of the heavy chain polypeptides and their carbohydrate content.

We can represent this basic structure by the general empirical formula, H_2L_2. Thus, a particular IgG1 molecule may be represented as either $\gamma 1_2\ \kappa_2$, or $\gamma 1_2\ \lambda_2$, depending on whether it contains kappa or lambda chains.

IgM and some IgA consist of multiple "IgG-like subunits". IgM consists of a pentamer of five such subunits, each of which consists of two mu chains and two light chains (either kappa or lambda); it therefore contains *ten* antigen combining sites, and is the largest of the immunoglobulins. IgA can exist either as a monomer or dimer (rarely a trimer) of

the basic H_2L_2 subunit, and therefore can bear two, four or six combining sites. IgM and polymeric IgA have an additional polypeptide associated with them, the *J-chain* (for "joining"). The J-chain is thought to participate in the process of polymerization of these molecules. (See also Chapter 9, Ig BIOSYNTHESIS)

DIFFERENT Ig ISOTYPES HAVE DIFFERENT BIOLOGICAL FUNCTIONS

The structures of the different immunoglobulin heavy chains are quite different from one another, in amino acid sequence, in chain length and in carbohydrate content. These differences result in marked differences in biological properties of the different **isotypes** (classes and subclasses) of Ig.

IgM Most efficient Ig at *complement fixation.*
Produced *early* in immune responses.
Serum IgM bears an additional polypeptide, the *J-piece* (for "*joining*"), as does polymeric IgA.
Serve as *membrane receptors* of B-cells (as "IgMs", an "IgG-like" monomer.

IgG *Most abundant* serum Ig.
Various subclasses can all *fix complement* (via classical or alternate pathway).
Can be *transferred across the placenta* into the fetal circulation.
Produced *late* in immune responses.
O*psonizing* activity via Fc receptors present on macrophages and PMNs.

IgA While IgA is the second-most abundant serum Ig, it is most characteristically a secretory immunoglobulin, and is the most abundant Ig in *exocrine secretions* (saliva, bile, mucus of the gut and respiratory tract, milk, etc.).
IgA in serum is mostly an α_2L_2 monomer; IgA in secretions is *polymeric* (mostly dimer, *i.e.* $[\alpha_2L_2]_2$, with some trimer), includes an *S-piece (for "secretory"),* and like IgM also bears the *J-piece*.

IgD Antigen-binding *receptor on B-cells* (together with IgMs).
Rare in serum; no other known biological function.

IgE Extremely low levels in serum.
Reaginic" antibody, responsible for *allergic reactions* following binding to surface of tissue mast cells (see Chapter 21).
While antibodies are generally very stable, IgE is an exception and is characteristically *heat labile*.

HOMOGENEOUS IMMUNOGLOBULINS: MYELOMA PROTEINS

We have already noted that *heterogeneity* is one of the most striking features of antibodies; this is understandable in that the primary function of antibodies is to bind to a wide variety of eiptopes. This heterogeneity, however, makes it difficult to carry out conventional structural studies on these molecules, particularly by amino acid sequencing or crystallization, which both require a homogeneous protein.

Our understanding of immunoglobulin structure resulted largely from the existence of MYELOMA PROTEINS, homogeneous forms of immunoglobulins produced by tumors of antibody-forming plasma cells. Since the tumors are monoclonal in origin, so are their protein products; in any particular patient, the monoclonal protein (also referred to as "M-component") is of a *single* heavy chain class and bears a *single* kind of combining site. One

reflection of their homogeneity is the fact that myeloma proteins are often *crystallizable*, unlike conventional Ig. In some cases the homogeneous protein consists of free light chain without an associated heavy chain; these free light chains are rapidly cleared by the kidneys and appear in the urine as BENCE-JONES PROTEINS. In other cases a tumor might produce only heavy chains which accumulate in serum, with no associated light chain.

A *variety* of pathological conditions can result in the presence of monoclonal Ig in serum, collectively referred to as **Monoclonal Gammopathies**. While some of these conditions are benign, many represent malignant tumors (cancers) of antibody-forming cells. Depending on their clinical presentation, these malignancies may be termed Multiple Myeloma, Plasmacytoma, Immunocytoma, Waldenstrom's Macroglobulinemia, Heavy Chain Disease or Light Chain Disease. We will use the general term "*myeloma protein*" to refer to *any* such monoclonal immunoglobulin.

IMMUNOGLOBULIN HEAVY AND LIGHT CHAINS HAVE VARIABLE AND CONSTANT REGIONS

Amino acid sequencing studies of Bence-Jones Proteins first showed that *light chains consist of a variable region and a constant region*. If several kappa-type B-J proteins (each from a different patient), are subjected to amino acid sequencing, one finds that the amino terminal half of the polypeptide (about 110 amino acids) shows substantial variation from one protein to another, while the carboxy-terminal half is essentially constant (except for *allelic variants* discussed in Chapter 6). Similarly, the heavy chains of different myeloma proteins have an amino-terminal variable region (also about 110 amino acids long), while the remainder of the polypeptide remains constant *within a given isotype*.

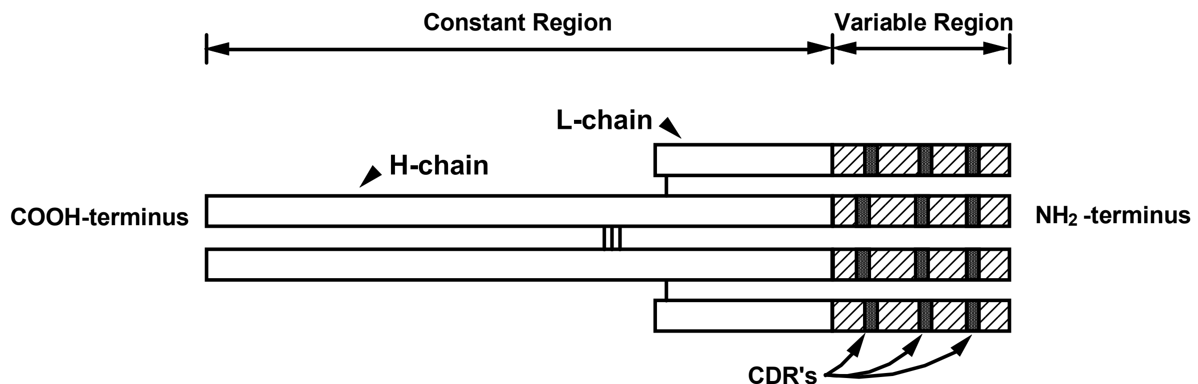


Figure 4-2

The antigen combining site of an antibody is made up of the variable regions of one light chain and one heavy chain. As can be seen in the diagram above, the two variable regions (V_H and V_L) are located in the F(ab) regions, precisely where we would expect them to be if they are responsible for antigen binding.

Within the variable regions, typically comprising 105-110 amino acids, some positions show more sequence variation than others. The highest degree of variability between different monoclonal proteins exists in three small sub-regions within the entire V-region, which were named the *Hypervariable Region***s**; the four segments outside these hypervariable regions are termed "framework" regions, and show considerably less diversity (although they are still "variable"). *The hypervariable regions form the antibody combining site in the native three-*

dimensional structure of the antibody, and are now commonly known by the more descriptive name of COMPLEMENTARITY-DETERMINING REGIONS (CDRs)

THREE FAMILIES OF V-REGIONS EXIST, one for *kappa* light chains, one for *lambda* light chains, and the third for *heavy* chains. In spite of their variable nature, one can readily determine from its amino acid sequence (even from the first few N-terminal residues) to which of these three families a given V-region belongs, kappa, lambda or heavy chain. However, in the case of V_H regions, one cannot predict which of the heavy chain isotypes it will be associated with; *all* heavy chains, regardless of class or subclass, draw from a *single pool* of V_H-regions. We will examine the cellular and genetic basis for this phenomenon in later chapters.

IMMUNOGLOBULIN HEAVY AND LIGHT CHAINS CONSIST OF GLOBULAR "DOMAINS "

Up to now we have diagrammed Ig molecules as consisting simply of linear polypeptides connected to one another by disulfide bonds. However, the polypeptides making up Ig molecules (and proteins in general) are not normally stretched out like pieces of spaghetti, but are folded into complex three-dimensional structures. In the case of a light chain, the V-region and C-region are each folded into separate, compact globular DOMAINS, the two of which are connected to each other by a short stretch of extended polypeptide chain. The heavy chain likewise has a single V-region domain, but has *several* C-region domains, a total of three in the case of IgG. The diagram in Figure 4-3 incorporates this more sophisticated view of immunoglobulin:

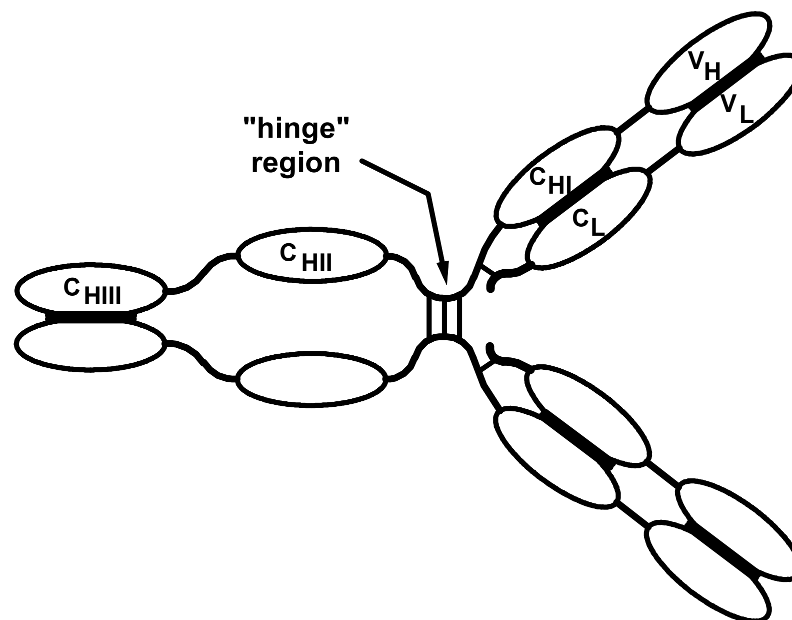


Figure 4-3

Every heavy or light chain is made up of one V-region domain and one or more C-region domains. In the case of light chains these are named V_L and C_L (Vκ and Cκ for kappa chains, *etc.*). In the case of heavy chains, these are termed V_H for the variable domain, and C_{HI}, C_{HII} *etc*. for the constant domains; for a particular heavy chain, *mu* for instance, the constant domains become $C\mu_I$, $C\mu_{II}$, etc.

Light chains have a single constant domain, heavy chains have three (IgG, IgD, IgA) or four (IgM, IgE) constant domains.

Each domain consists of a compact globular unit, and they are linked to one another by short stretches of extended polypeptide chain.

Different domains share varying degrees of amino acid sequence similarity and a high degree of similarity in their three dimensional structures. These patterns of similarity indicates that the different domains, and the three different immunoglobulin gene families (encoding the *heavy, kappa* and *lambda* chains), are all the product of a series of gene duplications of a primordial gene resembling a single domain.

Heavy chains have a stretch of extended polypeptide chain which is not part of any domain, between the C_{HI} and C_{HII} domains. This segment is known as the HINGE REGION, and is the region containing the cysteine residues involved in the H-H and H-L disulfide linkages.

COVALENT *AND* NON-COVALENT FORCES STABILIZE ANTIBODY STRUCTURE

Many kinds of molecular interactions contribute to the extraordinary stability of antibody structure. We have already seen that the chain structure is held together by a series of *covalent disulfide linkages* between the two heavy chains, and between each light chain and one heavy chain.

In addition, *powerful non-covalent interactions exist between various adjacent pairs of domains*, specifically between V_L and V_H, between C_L and C_{HI}, and between the two C_{HIII} domains; these are indicated by heavy lines between these regions in Figure 4-3. Thus, *even if all the disulfide bonds holding the chains together are broken, the overall structure of the Ig molecule will generally be maintained.*

Each domain forms an extremely stable globular structure, held together by powerful non-covalent forces as well as a single internal disulfide bond. The compactness of these structures confers a degree of resistance to proteolytic cleavage; it is for this reason that *much proteolytic* cleavage of immunoglobulins (as for instance with papain) takes place in the extended chains between domains.

ANTIBODIES BIND ANTIGENS BY POWERFUL *NON-COVALENT* FORCES

The basis of antibody-antigen binding is steric complementarity between the combining site and the epitope or antigenic determinant ("lock and key" concept). The combining site itself is made up by both V_L and V_H, most directly involving the hypervariable regions or CDRs ("complementarity determining regions") of both.

A variety of *non-covalent forces* are involved in this binding:

1) **Van der Waals interactions**. These are very weak and extremely short-range forces (they decrease with the seventh power of the distance), but they become important when many such interactions over the large area of an interactive surface are added together.
2) **Hydrophobic interactions**. Exclusion of water molecules between hydrophobic surfaces can also be a major contributor to antigen-antibody binding.

3) **Hydrogen bonds** and **ionic interactions** can also provide considerable stabilization of antibody-antigen binding, in those cases where the epitope is capable of participating in such interactions.

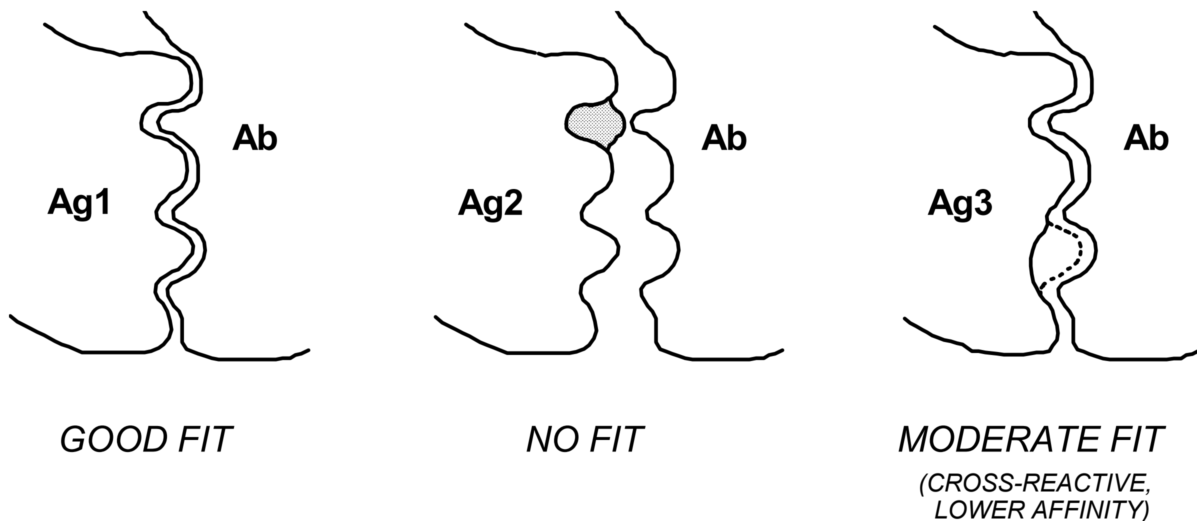


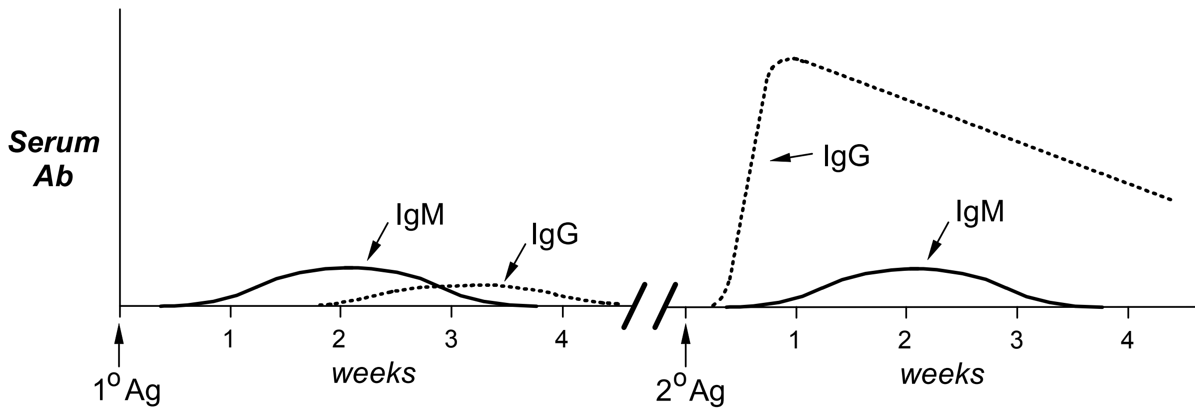
Figure 4-4

The single most critical factor in the strength of binding between antibodies and their antigens is the *degree of complementarity* ("goodness of fit") between the antibody combining site and the epitope (Fig. 4-4, left). A very slight change in the shape of either one can turn extremely strong binding into *no binding at all* (Fig. 4-4, center). Alternatively, a different kind of shape variation might turn strong binding into weaker binding, without eliminating it altogether; this is part of the basis of **cross-reactivity** between different but related antigens (Fig. 4-4, right).

IMMUNOLOGICAL MEMORY: PRIMARY *VERSUS* SECONDARY RESPONSES

One of the hallmarks of immune responses is their ability to display **memory**; the immune system in some way "remembers" what antigens it has seen previously, and responds *more effectively* the second time around. This is the basis, for instance, of acquired resistance to smallpox - having once recovered from the disease (or having been vaccinated), a person's immune system responds more rapidly the next time it is exposed to the virus, and eliminates it before the disease process can be initiated.

Having discussed the variety of known Ig *isotypes* and the concept of *affinity*, we can now identify several of the key features of immunological memory , illustrated in Figure 4-5 below, which shows the results of immunizing an animal with a "typical" antigen. The graph on the left represents the results of a *primary* immunization; plotted on the X-axis is the time after immunization in weeks, while the Y-axis represents a measure of the amount of antibody in the serum (which could be determined by any of the techniques we have described, agglutination, precipitation, ELISA, *etc*.). In the graph on the right we see the results following re-immunizing the same animal with the *same antigen* at some later date, termed a *secondary* immunization. The differences between the two graphs represent the features which define *immunological memory*.



PRIMARY AND SECONDARY HUMORAL RESPONSES

Figure 4-5

Outlined in the table below are the most important features which distinguish primary and secondary humoral immune responses.

PRIMARY RESPONSE	*SECONDARY RESPONSE*	
• SLOW • LOW Ab LEVELS • SHORT-LIVED	• FAST • HIGH Ab LEVELS • LONG-LIVED	*(kinetics of clonal selection)*
• MAINLY IgM • LOW AFFINITY	• MAINLY IgG • HIGH AFFINITY	*(Ig structure)*

The first three features are a consequence of the cellular events in immune responses, while the last two relate to the physical properties of the antibodies produced.

1) **Speed**. In a “typical” primary response we see a lag of about 4 days, followed by a slow rise of antibody to a peak at about two weeks. In the secondary, the lag is only two days, and antibody levels rise very rapidly to a peak within 4-6 days.

2) **Antibody levels**. The secondary response reaches peak antibody levels which are very much higher than in the primary.

3) **Duration**. Primary responses not only appear more slowly, they disappear fairly rapidly. Antibody titers in secondary responses remain at high levels for longer periods of time, and in humans may persist for many years.

4) **Ig class**. The majority of the antibody in a primary response is IgM, with some IgG appearing later in the response; in a secondary, IgG is the predominant antibody throughout the response. IgM also appears during a secondary response, but at levels

and with a time course comparable to the primary and is therefore swamped out by the higher levels of IgG. *IgM does not exhibit immunological memory*: it does not appear more rapidly or at higher levels in secondary responses, and does not undergo significant affinity maturation.

5) **Affinity**. The average affinity of antibody in a secondary response is higher than in a primary response. Even during the course of a particular secondary response, it can be shown that the affinity of "late" antibody is higher than that of "early" antibody. This progressive increase in the average affinity of antibody is known as AFFINITY MATURATION.

The net result of immunological memory is an immune response of greatly increased effectiveness. The antibody is made *more rapidly*, it is made in *higher amounts*, it *lasts longer*, and it is made with *higher affinity* so that it binds more effectively to its target. The mechanisms involved in all of these phenomena will become clearer when we have discussed the CLONAL SELECTION THEORY (see Chapter 7).

NOTE: It is important to keep in mind that immune responses vary greatly in their time course, intensity and persistence. While the figure above illustrates the antibody responses to a "typical" antigen in a "typical" organism, any particular response may be very different in many of its features. One antigen may result in a much slower or more rapid response than another, or may be unable to produce a secondary response at all. Nevertheless, the fundamental differences between primary and secondary responses are important and instructive generalizations.

CHAPTER 4, STUDY QUESTIONS:

1. Describe the structure and biological properties of the pepsin- and papain-generated PROTEOLYTIC FRAGMENTS of antibodies.
2. What are the distinguishing *structural* and *biological* features of each of the five CLASSES of human Ig?
3. What are the common structural features of *all* antibodies?
4. Why did the discovery of VARIABLE and CONSTANT regions of immunoglobulin heavy and light chains pose such a serious puzzle for molecular biologists?
5. How do the globular DOMAINS of immunoglobulins contribute to the remarkable stability of antibodies?
6. What features of SECONDARY antibody responses make them more effective than PRIMARY responses?

CHAPTER 5
COMPLEMENT

See APPENDIX (8) COMPLEMENT FIXATION ASSAY

> The complex of serum proteins known as COMPLEMENT plays key roles in the lytic and inflammatory properties of antibodies. The CLASSICAL pathway is initiated by antigen-antibody complexes (via complement components C1, C4, and C2), while the activation of the ALTERNATE pathway (via components B, D and P), and the MBLECTIN *("mannan-binding lectin")* pathway may be initiated by other substances independently of adaptive immune responses; *all three* pathways share those complement components involved in the inflammatory and lytic consequences, namely C3, C5, C6, C7, C8 and C9. The INFLAMMATION which is a consequence of complement fixation is illustrated by the manifestations of SERUM SICKNESS, and complement is also seen to be central to the normal process of clearing immune complexes, which is important in preventing IMMUNE COMPLEX DISEASE.

In the late 19th century, a researcher named Jules Bordet, following the earlier results of Richard Pfeiffer, was investigating the *lysis of the bacterium Cholera vibrio* (the agent which causes cholera) by immune sera, and found that the ability of an immune serum to lyse its targets was *lost upon heating* (*e.g.*, at 56° C for 30 min). This ability to cause lysis was also lost after simple storage of the serum for a few days at room temperature.

Bordet showed that such heating did not destroy the antibodies, however, since the addition of a small amount of normal, non-immune serum, to the heat-inactivated antiserum fully restored its capacity to specifically lyse cholera targets. Thus, the ability of immune serum to lyse bacteria depends not only on antibodies specific for *C. vibrio*, but also on a non-specific *heat-labile substance found in normal serum*. This substance became known as COMPLEMENT, since it "complements" the activity of the antibodies which are still present in heat-inactivated antisera.

> COMPLEMENT - A group of serum proteins which can be activated (= "*fixed*") by antigen-antibody complexes or other substances, which may result in lysis of a microbial target, or a variety of other biological effects important in both innate and adaptive immunity. (The majority of these proteins are produced by the liver.)

Before going into the details of the components of the complement cascade and their activation, let's preview the various **biological effects** which can be attributed to the action of complement, and identify those complement components or complexes which are responsible for these effects.

BIOLOGICAL EFFECTS OF COMPLEMENT

A) **Cytolysis** [**C$\overline{5b6789}$**] *(Note: the bar identifies an activated complex)*

Destruction of target cells by lysis of the cell membrane. This is termed **cytotoxicity** in the case of nucleated cells, **hemolysis** for red blood cells, or **bacteriolysis** in the case of bacteria. (NOTE: *Not all bacterial and eukaryotic cells are susceptible to complement- dependent lysis*).

B) **Anaphylotoxin activity** (= "vasoactive" or "phlogistic") [**C3a, C5a**]

Stimulation of mast cells to release histamine and other substances, resulting in increased capillary permeability and local accumulation of fluid in the tissue.

C) **Chemotaxis** [**C5a, C$\overline{5b67}$**]

Attraction of polymorphonuclear neutrophils (PMN's) to a local site of inflammation.

D) **Opsonization** (= "immune adherence") [**C3b**]

Facilitation of phagocytosis by macrophages or PMN's via cell-surface receptors specific for complement components ("complement receptors", or "CRs")

E) **Tissue damage** [**C$\overline{5b6789}$; PMN's**]

Both the lytic complex and the inflammatory PMN's can cause considerable damage to normal tissues, for instance in an Arthus Reaction or in Immune Complex Disease.

The consequences of complement activation can be categorized into two general classes:

- ***Facilitating antibody function***; destruction and removal of foreign material.
  - Target cell *lysis;* the lytic *"membrane attack complex" ("MAC")* can be produce by all three of the complement pathways discussed below.
  - *Removal of immune complexes* ("*immune clearance*"); this is a critically important function facilitated by the presence of receptors ("CR") for various complement components on the surface of leukocytes and erythrocytes. The special process by which *soluble* (*i.e.* small) immune complexes are normally cleared from serum relies on the presence on erythrocytes of CR1 complement receptors which are capable of binding C4b (this process is discussed later in this chapter).
- ***Development of inflammation***; increased circulation and *accumulation of fluids and cell*s all contribute to the cardinal signs of inflammation (heat, redness, swelling and pain); these functions are mediated directly and indirectly by proteolytic products of the complement cascade (C3a, C5a).

THREE PATHWAYS FOR COMPLEMENT FIXATION

The process of complement fixation requires specific *protein/protein interactions*, it involves *proteolytic cleavages* and conformational changes of proteins, and *new biological activities* are generated as a result.

Three distinct (although related) mechanisms are known which can initiate the complement cascade, the **Classical Pathway**, the **Alternate Pathway**, and the more recently recognized **MBLECTIN Pathway**. The central event in all three of these modes of complement activation is the cleavage of component C3. The pathways differ only in the mechanism by which they achieve this cleavage, and we will consider them in turn.

CLASSICAL PATHWAY: ANTIBODY-DEPENDENT COMPLEMENT FIXATION

This pathway is initiated by *antigen/antibody complexes* and requires **heat-sensitive** complement components. An outline of the components and events in complement fixation by the classical pathway is shown in Figure 5-1. Let's examine in some detail the reactions involved.

1) **Ag + Ab → AgAb**

(S + A → SA) An antibody/antigen complex is formed.

Conventional designations for Ag and Ab are ***S*** (for antigenic **S**ite) and ***A*** (for **A**ntibody). While many different forms of antigen can fix complement and cause its various biological consequences, the concept of lysis is only meaningful for *membrane-bound antigens*; therefore, in the discussion below, we will assume we are dealing with an antigen in a membrane susceptible to lysis (as, for example, an erythrocyte or a gram-negative bacterial cell).

2) a) **C1q, C1r, C1s** $\xrightarrow{Ca^{++}}$ **C1qrs** (Spontaneous; occurs in serum without AgAb complex.)

b) **SA + C1qrs → SA C$\overline{\text{1qrs}}$** Requires either a single IgM or 2 adjacent IgG's.

The antigen-antibody complex binds C1qrs and activates the esterase activity associated with C1s.

(For simplicity in the discussion below, we will leave out the "SA" (or AgAb), understanding however that it is required for the *initiation* of this pathway and is associated with the early complexes. The bar over the C1qrs is a conventional way of indicating an *activated complex*.)

3) **C$\overline{\text{1qrs}}$ + C4 → C1$\underline{\text{qrs/4b}}$ + C4a**

new active complex (minimally vasoactive)

C1s (in the complex) binds soluble C4 and cleaves it into C4a and C4b; C4b becomes covalently bound to the complex or to the nearby membrane (where it has minimal opsonizing activity); C4a is released, and while it is somewhat vasoactive, its low level of activity is probably not biologically important.

Some **amplification** of the process occurs here, since dozens of C4b molecules can be generated and bound to the membrane.

CLASSICAL COMPLEMENT PATHWAY

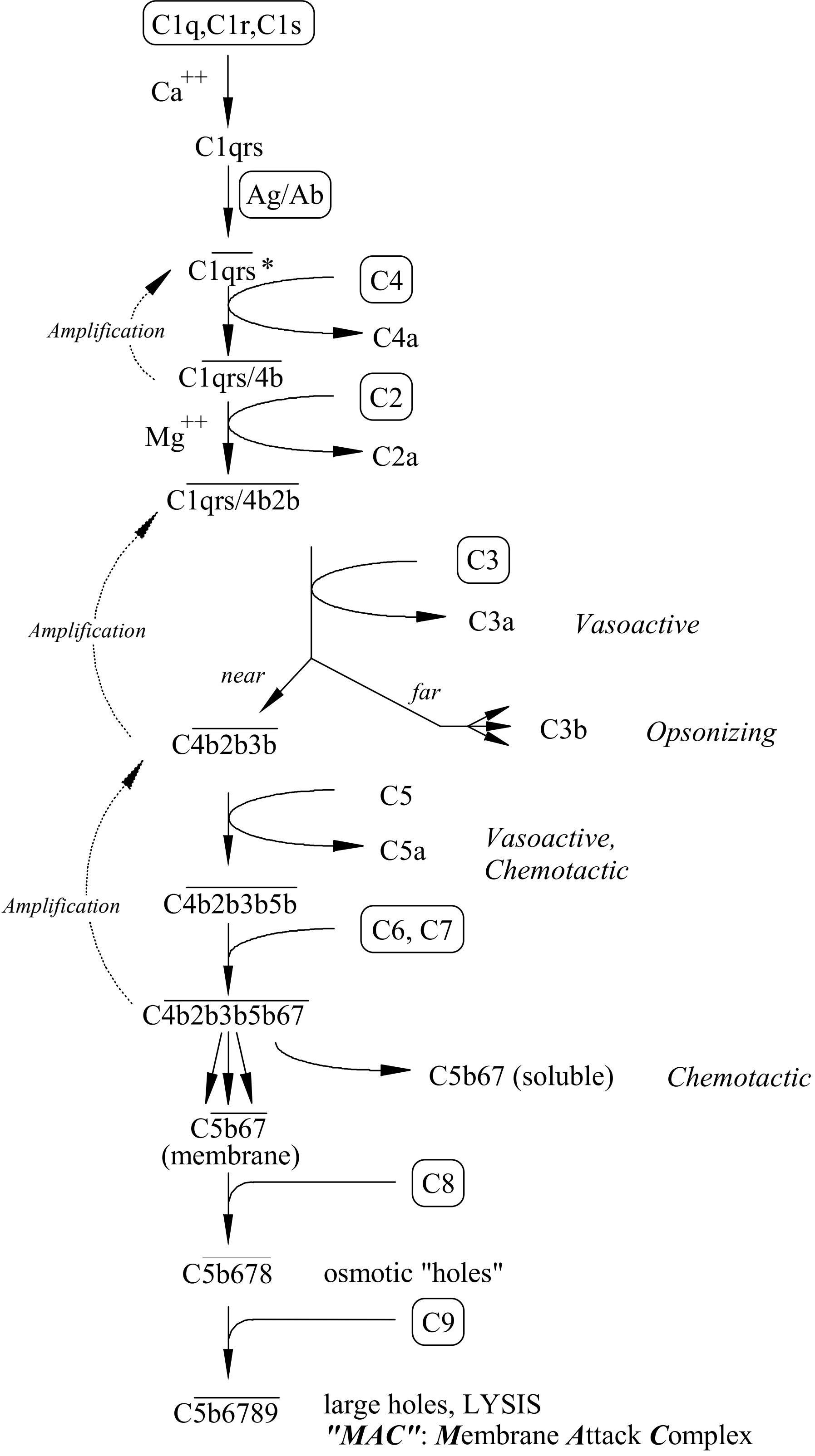
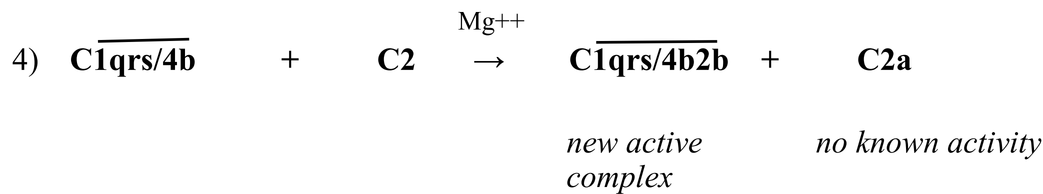
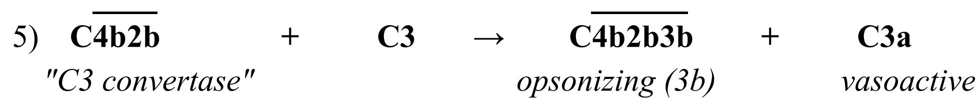


Figure 5-1



The esterase activity of C1s acts again, this time to cleave C2 into C2b (which is bound by 4b in the complex) and C2a (which is released). (NOTE: *The names of 2a and 2b are reversed from the usage you may find in older literature as well as some current sources. Using the present nomenclature, all those fragments which bind to the complex are named "b", while those that are released in soluble form are named "a"*.)

At this point the presence of the AgAbC1qrs complex is no longer necessary for the cascade to continue (even though it's still present), and we'll omit it in the diagrams below.

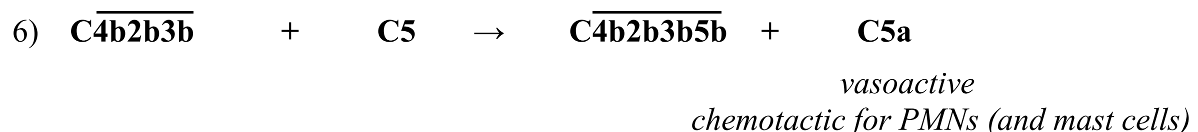


The C4b2b complex has an enzymatic activity called **C3 convertase**, indicating it can cleave C3 into C3a and C3b. This is a *key step* in this pathway, involving *biological amplification* and the generation of molecules with *new biological activities*.

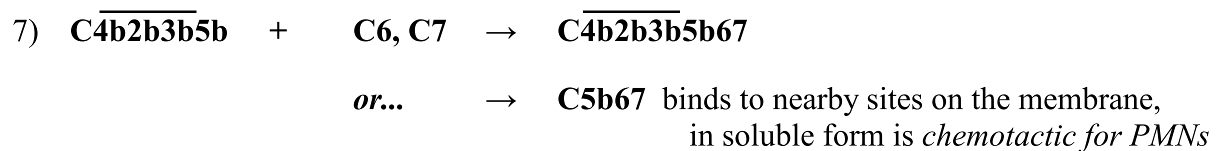
Many hundreds of C3 molecules can be cleaved by a single C4b2b complex, resulting in considerable **amplification**. The resulting C3b molecules can either be *bound by the complex* or can be released and *bind elsewhere* on the membrane (C3b molecules which are *not* bound in one place or the other are rapidly destroyed.)

C3b has powerful opsonizing activity, whether it is bound in the complex or to some other site on the membrane.

C3a is strongly vasoactive. It is also chemotactic for mast cells.



Cleavage of C5 results in a new complex and another biologically active molecule, C5a, which is both **vasoactive and chemotactic**.



The C5b67 complex may be released and bind to a nearby site in the membrane, and in soluble form also exhibits chemotactic activity. The resulting C4b2b3b complex left behind can then sequentially bind additional C5, C6 and C7 molecules, which results in *another important site of amplification in the complement cascade*.

The final reactions of the complement cascade produce the membrane-destroying complexes:

8) **$C\overline{5b67}$** + **C8** → **$C\overline{5b678}$**
osmotic "holes", leakage

9) **$C\overline{5b678}$** + **C9** → **$C\overline{5b6789}$**
Membrane Attack Complex, “MAC”, large "holes", lysis

The formation of large, open channels in target cell membranes results in the loss of hemoglobin from erythrocytes (visible as HEMOLYSIS) or leakage of cytoplasmic contents and death of nucleated cells (CYTOTOXICITY).

ALTERNATE PATHWAY OF COMPLEMENT FIXATION

The biochemist Louis Pillemer discovered in the 1950's that complement fixation could be triggered by the yeast polysaccharide Zymosan in the *absence* of antibody, by a process which has become known as the **Alternate Pathway**. The initial steps of this process are quite different from those of the classical pathway and involve several unique serum components, namely factor P (for "properdin"), and factors named B and D. The mechanism is outlined below, and the initial step relies on the fact that *very small amounts of soluble C3b are normally present in serum*, due to low levels of spontaneous C3 cleavage (“tickover”), which may not be C4-dependent.

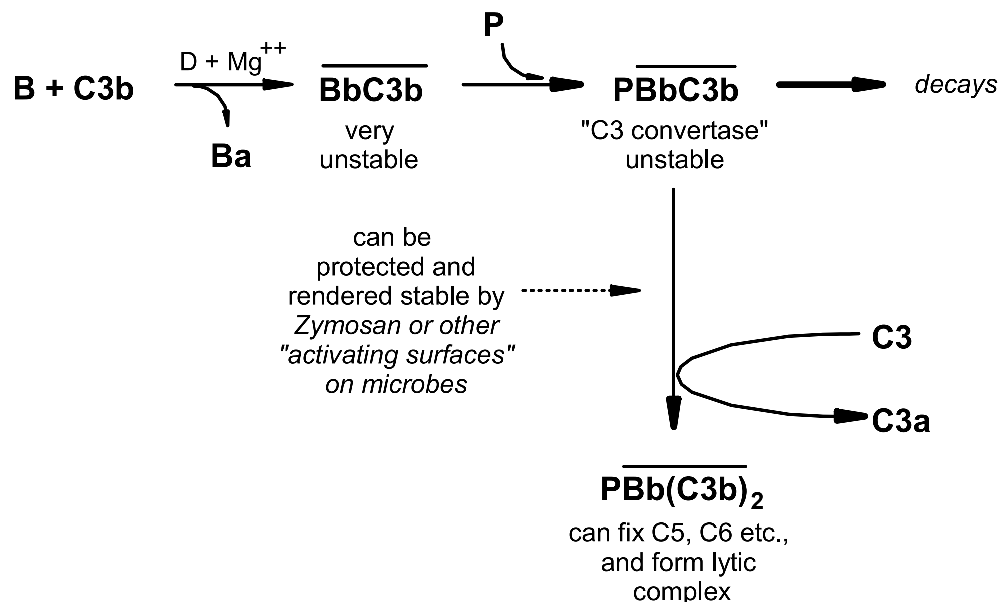


Figure 5-2

The initial steps of the alternate pathway result in the formation of an *unstable* complex which has "C3 convertase" activity (namely PBbC3b). This complex is formed continuously at low levels and rapidly degrades, but it may be bound and stabilized by "activating surfaces" such as *zymosan*-containing yeast cell walls, *LPS*-containing gram-negative bacteria, *teichoic acid*-containing gram-positive bacteria, and others. The result of stabilization is that *these C3-convertase complexes can carry out the remainder of the complement fixation cascade* in a manner identical to what we have outlined above for the classical pathway.

Thus, fixation of complement by the alternate pathway can yield all the variety of biological activities we have already seen -- chemotaxis, opsonization, anaphylotoxic activity and lysis (only if the membrane is susceptible to lysis, of course), in addition to all the inflammatory sequelae.

Initiation of the alternate pathway can be triggered by the presence of a variety of **microorganisms** (as mentioned above) and **parasites**. In addition, **aggregated immunoglobulin** may also trigger the alternate pathway, even isotypes of Ig which are incapable of fixing complement by the classical pathway (such as human IgG4 and IgA).

RELATIONSHIP BETWEEN CLASSICAL AND ALTERNATE PATHWAYS

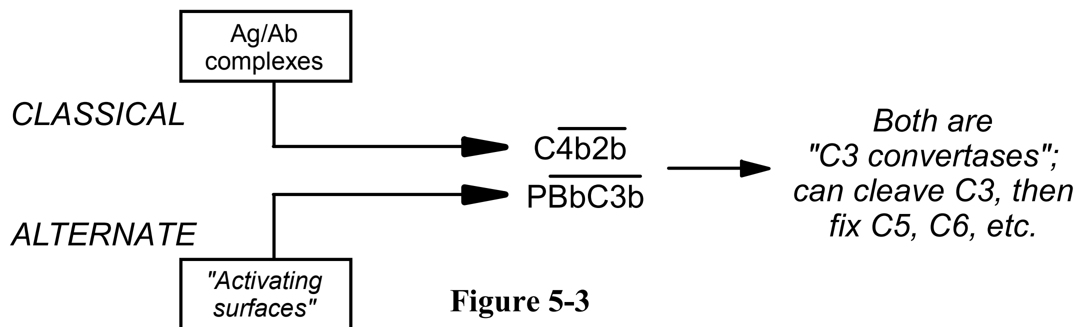


Figure 5-3

Figure 5-3 illustrates the relationship between the two complement pathways which we have just discussed. Both the classical and alternate pathways, using *different* mechanisms, generate complexes which have *C3 convertase activity*. After that point the two pathways are identical - each complex can generate and bind a new C3b molecule, then proceed to fix C5, C6 *etc*. It is worth noting that while the initiation of the alternate pathway is dependent on Mg^{++} (although this may not be absolute), the early steps of the classical pathway are dependent on *both* Mg^{++} and Ca^{++} (see steps 2 and 4 in the classical pathway diagram).

MBLECTIN COMPLEMENT PATHWAY

A third mechanism for the initiation of complement fixation has been described which depends on the presence of another normal serum protein known as the ***m**annan-**b**inding lectin*, or MBLECTIN. This protein is capable of binding to microbial carbohydrates containing terminal mannose residues, and consequently binding two other proteins, MASP-1 and MASP-2 (**m**annan-binding lectin-**a**ssociated **s**erum **p**rotease-1 and -2). The resulting complex has C4-convertase activity (*i.e.* it can bind and cleave C4), and the remainder of the

complement cascade (C2, C3, C5 *etc.*) is activated just as in the case of the classical and alternate pathways. This distinct complement pathway is diagrammed in Figure 5-4.

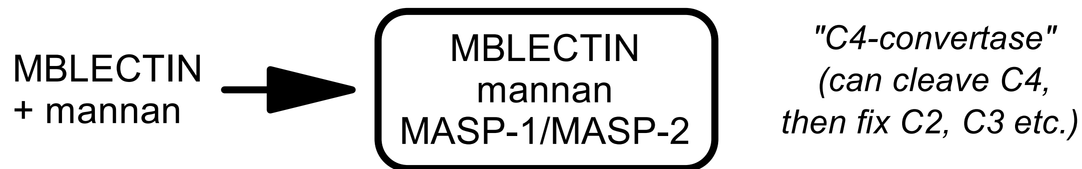


Figure 5-4

SIGNIFICANCE OF THE NON-CLASSICAL COMPLEMENT PATHWAYS: *RAPID, NONSPECIFIC ACTION*

Since they do not require the presence of antibody, both the ALTERNATE and MBLECTIN pathways can be *initiated much more rapidly* than the classical pathway upon initial exposure to a microorganism. There is no need to wait for several days while antibody formation is initiated, and it is thus a manifestation of "innate" immunity. On the other hand, these pathways do not have the exquisite specificity conferred by antibodies, and they are limited to recognizing only certain kinds of microbial structures for triggering (these are both characteristic features of innate immunity). Nor do they exhibit any form of memory.

Nevertheless, the alternate pathway, at least, can *augment* the biological effectiveness of specific antibodies (produced by *adaptive immunity*) in at least two ways. *First*, as mentioned above, aggregated Ig's (for instance, clustered on the surface of a microorganism) may trigger the alternate pathway, regardless of whether the classical pathway has also been initiated. *Second*, the C3b molecules generated by the classical pathway can promote the formation of the alternate pathway's "C3 convertase", PBbC3b, again resulting in increased complement fixation.

BIOLOGICAL EFFECTIVENESS OF COMPLEMENT DOES NOT DEPEND SOLELY ON LYSIS

While complement fixation is generally thought of as culminating in lysis, we have already noted that only a limited variety of bacteria (mostly gram-negative organisms) are susceptible to such lysis (and not all nucleated cells, for that matter). This does not mean, however, that such cells are completely resistant to the consequences of complement fixation. A gram-positive bacterium can still be effectively *opsonized* (and consequently phagocytosed and destroyed) as a consequence of complement fixation by either pathway. In addition, the *inflammatory* sequelae of complement fixation (increased blood flow and accumulation of scavenger cells) all tend to increase the effectiveness of microbial destruction.

The final outcome following introduction of a pathogen into an organism will depend on many factors, including its susceptibility to complement dependent lysis and opsonization and its ability to trigger the alternate pathway of complement, as well as on the nature of the *adaptive* immune response which it generates (depending on its degree of immunogenicity and the isotype distribution of the resulting antibodies) and possible previous exposures of the immune system to the same or similar pathogens (immunological memory). Overall, the **opsonizing and inflammatory effects of complement appear to be more significant than lysis** in providing protection against infectious organisms.

REGULATION OF THE COMPLEMENT CASCADE

We have thus far mentioned only those factors which *activate* complement components and drive the reactions in the direction of biological effectiveness. If these were the *only* relevant factors, then an initial complement fixation event would result in an uncontrolled inflammatory response and the consumption of all the complement available in the system; clearly this does not normally occur.

A variety of factors exist which are responsible for the *inactivation* of the various complement products which are biologically active. Some of these factors are known, including ones which inactivate the C1 complex, the C3a, C3b, C4a and C5a fragments, and the C5b67 complex. These inhibitory factors are critical to the normal balance of the complement system. For example, a hereditary deficiency in C3b-inactivator (called "Factor I"), results in excessive breakdown of C3 by the BbC3b complex and greatly depletes the serum levels of C3, which in turn results in high susceptibility to recurrent bacterial infections. A great deal is known about mechanisms which regulate complement activation, although we will not discuss them further here.

IMMUNE COMPLEXES: COMPLEMENT-MEDIATED TISSUE DAMAGE

One of the consequences of antibody binding to its antigen is the elimination of the Ag/Ab complex by phagocytic cells; removal of foreign antigens is obviously one of the main goals of the immune response. If antigen/antibody complexes are formed in the circulation, however, they may have other consequences which can be quite harmful to the host.

Antigen/antibody complexes in the bloodstream can bind to the basement membranes of blood vessels and kidney glomeruli; at these sites they can fix complement which results in damage to tissues. This is the basis for IMMUNE COMPLEX DISEASE. The antigen may be associated with a pathogen (a virus, for instance), it may be clinically introduced (with blood transfusions or with drug or antibody therapy), or may be a normal "self" component (in the case of AUTOIMMUNE DISEASES such as RHEUMATOID ARTHRITIS and LUPUS ERYTHEMATOSUS).

Immune complexes are harmful only if they fall within a limited range of sizes. If the complexes are very large, they are efficiently removed by phagocytic cells (*e.g.* liver Kupfer cells and lung macrophages); if they are very small, they are effectively soluble and are not trapped in basement membranes (and these small complexes are normally removed by erythrocytes, discussed below). Immune complexes which fall between these two extremes, however, may become trapped in the basement membranes of the vasculature in various organs where they can fix complement and cause tissue damage.

The consequences of deposition of complexes within tissues include local inflammation and tissue necrosis. These are the consequences of the activity of the various complement components which we have already outlined, and there can be more systemic symptoms such as fever and malaise.

Let's illustrate this with a classic example known as **One-Shot Serum Sickness**. A man having suffered a severe tetanus-prone wound is given a single injection of horse serum containing anti-tetanus toxoid. After about a week, he complains of fever, malaise and rashes, which spontaneously subside after another week; and he remains well after that. To understand the progress of this reaction, we will refer to Figure 5-5 below:

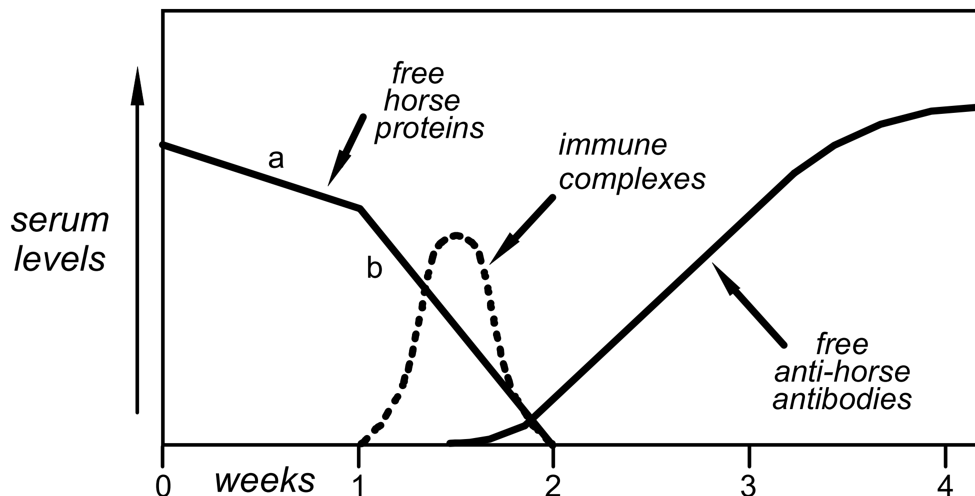


Figure 5-5

During the first week following the injection, the horse proteins are removed from the circulation at a slow rate determined by their biological properties; this is referred to as **Biological Clearance** ("*a*"). At the beginning of the second week, they begin disappearing at a more rapid rate; this is due to the production of anti-horse antibodies and a resulting **Immune Clearance** of the complexes ("*b*"). Later on (in the third week) we can find appreciable levels of free circulating anti-horse antibodies, but only after the horse proteins themselves are gone.

During the period of immune clearance, at a time when *both* horse protein *and* anti-horse antibodies are present, complexes may be formed which are of the appropriate size to be trapped in basement membranes; and the results include the rash (local necrosis) as well as the fever and other systemic manifestations. *In this example the disease is self-limiting*, since there is no continuing source of antigen -- when the horse serum proteins are cleared, the symptoms disappear. However, *if there is a continuing source of antigen*, immune complex disease may present a more serious chronic problem; for example, the antigen may be associated with a chronic viral infection (*e.g.* hepatitis), or may be a normal tissue component (in the case of AUTOIMMUNITY).

This illustrates the danger of any therapy which makes use of foreign proteins, including anti-toxin therapy, and blood transfusions in general. This example is called "*one shot*" because it involves a single *primary* exposure to the antigen, and the disease symptoms are therefore delayed a week or two while induction of antibody formation takes place; if circulating antibody is already present at the time of injection, the disease will appear much more rapidly. It can be particularly dangerous to treat someone intravenously with a protein to which he has already been exposed, (with a *second* course of horse serum anti-toxin, for instance) due to the presence of pre-existing antibody and the induction of a rapid and powerful secondary antibody response.

C1 & C4 COMPLEMENT DEFICIENCIES: INADEQUATE CLEARANCE OF IMMUNE COMPLEXES

We can learn much by examining the clinical *consequences of congenital complement deficiencies*. Deficiencies of components of the membrane attack complex (C5-9), as well as the alternate pathway components B, D and P have the expected results of increasing susceptibility to bacterial infections. More surprising, however, is the fact that deficiencies of C1 and C4 result in increased susceptibility to the development of *autoimmune disease* (SLE, or Systemic Lupus Erythematosus, which will be discussed later), and associated *immune complex disease*.

This reflects the fact that *C1 and C4, unlike C3 and the later components of the classical pathway, can be efficiently "fixed" by* <u>soluble</u> *Ag/Ab complexes*, and these components are therefore critically important in causing the rapid removal of such complexes from the circulation. This process depends largely on the presence of membrane receptors for C4b on erythrocytes (which express the complement receptor CR1). Soluble immune complexes which contain C4b therefore bind efficiently to RBCs, which carry them to the liver where the complexes are stripped off by macrophages, and the RBCs are returned unharmed to the circulation. When these early complement components are absent, small immune complexes which are normally harmless (because they are rapidly removed) may grow in size and eventually be deposited in tissues, and the alternate pathway components can then promote the inflammatory consequences we have already discussed. The resulting tissue damage can feed a continuing cycle of formation of further immune complexes and induction of autoimmune antibodies (see Chapters 18 and 19 on TOLERANCE and AUTOIMMUNITY).

The importance of this mechanism for clearing immune complexes results from the fact that *normal serum always contains small amounts of immune complexes,* mostly due to the spontaneous formation of autoantibodies to various "self" components. In the presence of the normal complement-dependent clearing mechanism, these autoantibodies and immune complexes are harmless. When this mechanism is defective, these immune complexes may grow and fuel a continuing cycle of tissue damage and autoimmunity (see Chapter 19).

CHAPTER 5, STUDY QUESTIONS:

1. What is required for the initiation of complement fixation by each of the CLASSICAL, ALTERNATE and MBLECTIN pathways? What do the three pathways have in common?
2. Which proteolytic fragments of complement components have known autonomous biological activities?
3. What are the clinical consequences of the congenital absence of various complement components?
4. What medical situations are likely to result in ONE-SHOT SERUM SICKNESS?
5. What is the process by which soluble immune complexes are normally cleared from the blood?

CHAPTER 6
ANTIBODY GENETICS: ISOTYPES, ALLOTYPES, IDIOTYPES

See APPENDIX: (3) OUCHTERLONY; (4) AFFINITY CHROMATOGRAPHY

Human immunoglobulins are made up of LIGHT and HEAVY chains encoded by a total of 14 CONSTANT REGION GENES organized into *three gene families*, namely ONE KAPPA gene, FOUR LAMBDA genes and NINE HEAVY CHAIN genes. Allelic variants exist for the *kappa locus* and six of the nine *heavy chain loci*.

The nine heavy chains define nine human CLASSES and SUBCLASSES of human immunoglobulin. Together with the five different light chains, these genes represent 14 distinct ISOTYPES which are present in *all* normal human sera. ALLOTYPES, or allelic variants within the constant regions, are known to exist for some of these isotypes, and are inherited in a Mendelian co-dominant fashion. IDIOTYPES consist of the unique combination of V_H and V_L which characterize a particular immunoglobulin's *combining sites*.

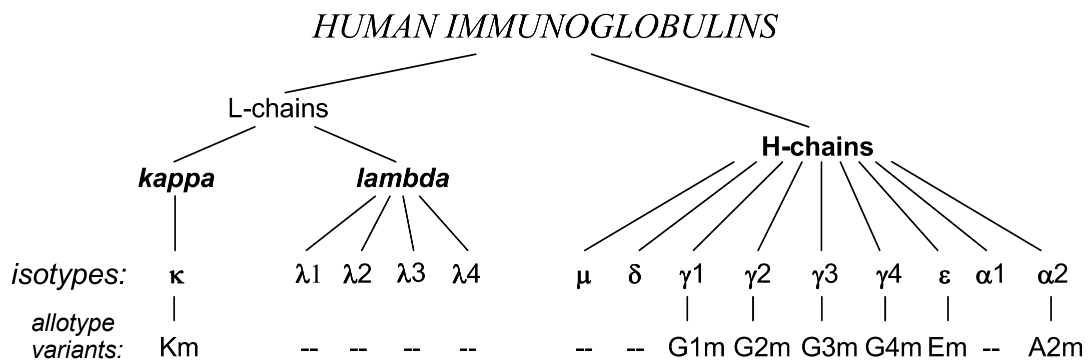


Figure 6-1

Immunoglobulins all have light and heavy chains, but the light and heavy chains of a particular Ig molecule may be different from those of other molecules. We have already seen that different kinds of heavy chains define the *class* (and *subclass*) of antibody to which they belong. We have also learned of the two varieties of light chains that exist, *kappa* and *lambda*. These and other structural differences between Ig polypeptides can be organized into three categories of differences, which we call *isotypic, allotypic*, and *idiotypic*. These are commonly distinguished serologically, *i.e.*, by the use of antibodies which recognize different specificities on target immunoglobulins.

ISOTYPES -- Distinct forms of light or heavy chains which are present in *all* members of a species, encoded at distinct genetic loci. *Kappa* and *lambda* are *isotypes* of light chains. Mu (μ), delta (δ), gamma-1 (δ1), *etc*. are *isotypes* of heavy chains. *All* isotypes can be readily found in *all normal sera*.

ALLOTYPES -- Genetic variants within the C-region sequences of particular isotypes that are inherited in an allelic manner (*"allelic type"*). Different members of a species will therefore *differ* from one another with respect to which particular alleles of a given isotype they received from their parents. Km1 and Km2 are *allotypes* of humans kappa chains; G1m(4) and G1m(17) are *allotypes* of human gamma-1 chains. The presence of particular allotypes, like isotypes, can be readily detected in those normal sera in which they are present.

IDIOTYPE -- An antigenic specificity (epitope) which distinguishes a particular *combination* of V_H and V_L (the antigen recognition site) from all others. Thus, a particular monoclonal immunoglobulin (a myeloma protein, for example) will bear an idiotype *different from any other*. Unlike isotypes or allotypes, particular *idiotypes* can generally be detected (with very rare exceptions) only in sera from myeloma patients. This is because any particular idiotype will be represented only at extremely low levels among the many thousands of kinds of combining sites present in serum immunoglobulin, even in specific immune responses.

Let's go through a series of experimental analyses of human myeloma proteins and Bence-Jones proteins. We will use classical serological approaches to demonstrate:

(1) The existence of two light chain isotypes (κ and λ);

(2) The presence of two κ chain allotypes (Km1 and Km2;

(3) The existence of two λ chain isotypes (λ1 and λ2).

EXPERIMENTAL DEFINITION OF LIGHT CHAIN ISOTYPES: KAPPA AND LAMBDA

We'll begin with a series of eight *purified Bence-Jones proteins*, each from a different patient (labeled A through H), and carry out an antigenic analysis to illustrate the properties of *isotypes* and *allotypes*. Let's take four of these proteins (A,B,C and E in this example) and use each them to immunize rabbits, resulting in four antisera. We will then take each of the four antisera (labelled "Ab") and test each of them by double diffusion in agarose (see APPENDIX 3, OUCHTERLONY analysis) against each of the eight original proteins. In the table below, a *plus* indicates that the antibody *does* show a line of precipitation with the particular B-J protein, and a *minus* that it *does not*.

Bence-Jones Proteins

Ab	A	B	C	D	E	F	G	H
Rab α A	+	+	−	+	−	+	+	−
Rab α B	+	+	−	+	−	+	+	−
Rab α C	−	−	+	−	+	−	−	+
Rab α E	−	−	+	−	+	−	−	+

Table 6-1

Clearly, while we have *four* different antisera, we see only *two* different patterns of reactivity, since antisera made against A and B show the same pattern as each other, and those against C and E are also the same. The results indicate that these Bence-Jones proteins can be separated into two "kinds": one group (A, B, D, F and G) reacts only with one pair of antibodies (αA and αB), the other group (C, E and H) reacts only with the other pair (αC and αE). We could call these two kinds of B-J proteins types I and II, or types *a* and *b*, but they have actually been named ***Kappa*** and ***Lambda*** after their discoverers, Korngold and Lipari.

Thus we find that *all* B-J proteins (and, in fact, all human immunoglobulin light chains) can be classified as either kappa or lambda type. Now we ask whether these two varieties represent *isotypes* or *allotypes* of light chains? To answer this question we must use our antisera to test a large number of normal human sera; when we do this we find that **every normal serum contains both kappa chains and lambda chains**. Therefore, these two types of light chains **fulfill the definition of isotypes**. This implies that there are two separate genetic loci which encode these two light chain types - they are *not* encoded by two different alleles at a single locus.

Kappa and lambda light chains are both found in immunoglobulins of all mammals. In humans about 60% of all light chains are of kappa type, and 40% are lambda. In mice and rats, on the other hand, about 95% of light chains are kappa type, while the vast majority of light chains of horses and cattle are lambda-type. The biological significance of these differences is not known.

The various *heavy chain classes and subclasses* which have been listed in Chapter 4 are all *isotypes* of human heavy chains. Unlike light chain isotypes, however, heavy chain isotypes are known to differ from one another in a variety of important biological functions -- IgM is the most efficient at complement fixation, IgA is most efficiently secreted into exocrine fluids, *etc*. (see Chapter 4). [It should be noted that different vertebrate species have different numbers (and kinds) of heavy chain isotypes. Rabbits, for example, have only a single kind of IgG (*i.e*. they have no gamma-chain subclasses), while the major serum immunoglobulin in chickens is termed IgY.]

EXPERIMENTAL DEFINITION OF KAPPA CHAIN ALLOTYPES (InV/Km)

Let's analyze this collection of B-J proteins further. We now take our Rabbit-anti-B (which is one of our anti-kappa reagents) and test it again in Ouchterlony against four of the kappa type chains, A, B, D and F. This time, however, we will put the four antigens in nearby wells to examine their antigenic relatedness (as opposed to simply testing each one independently for its ability to precipitate with the antibody). The results are shown below:

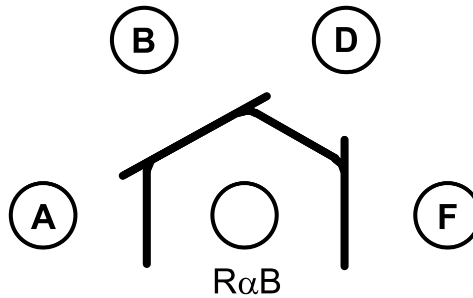


Figure 6-2

While Rabbit *anti-B* reacts with all four antigens, it clearly detects *more* epitopes on two of them, B and F, than on the other two. That is to say, in addition to detecting an epitope common to all of them, the antibody is detecting at least one epitope present on B and F which is *not* present on A and D.

In order to make the analysis clearer, we will **absorb** the antiserum, removing those antibodies which react with all four proteins and leaving only those antibodies reacting specifically with B and F. We do this by passing the antiserum over an "immunoadsorbent" column which contains antigen A covalently coupled to a matrix (see AFFINITY CHROMATOGRAPHY in APPENDIX 4). All those antibodies which can bind to B-J protein "A" will do so, all the others will pass through the column and will be recovered. The recovered antiserum (which passes through the column), we call RαB[abs A] . When we test this *absorbed* antiserum in the same manner as before, we see the results below:

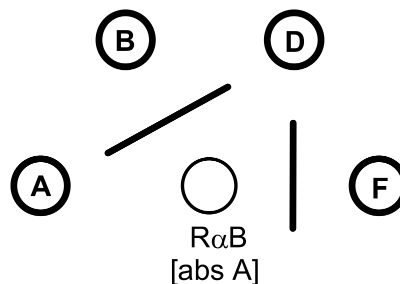


Figure 6-3

This absorbed antiserum now reacts *only* with B-J proteins *B* and *F*, and not at all with *A* and *D*. It therefore defines *two different forms of kappa chains*, which, when they were discovered, were originally called Inv^{+} and Inv^{-}.

Now we need to ask once again, are these variants *isotypes* or *allotypes* of kappa chains? When we use this specific antiserum to test a large number of normal human sera, we find that *only some sera* show a reaction. Furthermore, we find that the presence or absence of this epitope (defined by Rab α B[abs A]) can be accounted for by the segregation of a pair of Mendelian alleles at a single locus (this information comes from family studies). This Inv factor is therefore *not* an isotype, but fulfills the definition of an *allotype*.

Thus, every individual human has kappa chains (an "*isotype*"), but the kappa chains in any individual's serum may exist as either or both of at least two allelic forms ("*allotypes*"); some people have only Inv^{+} kappa chains, others have only Inv^{-} kappa chains, and some (who are heterozygotes) have both. Modern nomenclature replaces the name Inv with **Km** (for Kappa-marker), and a total of *three* allelic epitopes are now known to be defined by the Km locus, named Km(1), Km(2) and Km(3). Our anti-Inv antibody (Rab α B[abs A]) represents an *anti-Km(1)* antibody, and by a similar set of immunizations and absorptions we can produce specific antisera detecting *Km(2)* and *Km(3)*. Using these antibodies to study human populations, we can review the relationship between genotype and phenotype for the three alleles defined by this set of antisera, illustrated as follows:

Phenotype versus Genotype for Kappa Allotypes

Genotype	Phenotype
Km(1/1)	Km(1+)
Km(1/2)	Km(1+2+)
Km(1/3)	Km(1+3+)
Km(2/2)	Km(2+)
Km(2/3)	Km(2+3+)
Km(3/3)	Km(3+)

Since kappa chain alleles are *codominantly* expressed in all people (as is the case for *all* immunoglobulin genes), an individual who is heterozygous for the Km(1) and Km(2) genes will will always show a *positive* result when his serum is typed using either reagent. Thus, there are three alleles at the single *kappa* locus, and a total of six unique genotypes and phenotypes.

Many of the *human heavy chains* also show allelic variants. "Gm" is a general term for allotypes of human gamma chains (meaning "***G***amma ***m***arker"). "G1m", for example, specifies allelic markers of *gamma-1 heavy chains* which exist in several allelic forms, two of which are known as G1m(4) and G1m(17). Similarly, G3m specifies allelic markers of gamma-3 heavy chains, *etc*. As another example, while all normal human sera contain both IgA1 and IgA2 (which are therefore *isotypes*), the alpha-2 heavy chain present in IgA2 can exist as either of two allelic variants, which are known as A2m(1) and A2m(2).

Immunoglobulin allotypes have been known since the 1950's, with the discovery of rabbit allotypes by Oudin and of human allotypes by Grubb. Most of what we know of the remarkable organization of immunoglobulin genes, culminating in their characterization in the early 1980's by recombinant DNA techniques (which we will discuss in Chapter 8), was based on studies of the classical genetics of immunoglobulin allotypes in humans, rabbits and mice.

EXPERIMENTAL DEFINITION OF ISOTYPES OF HUMAN LAMBDA CHAINS (Oz)

Let's carry out for a third time the kind of analysis we just described for kappa chains, this time with the *lambda chain* Bence-Jones proteins in the panel above. We will use Rabbit *anti*-E (antiserum against one of the lambda chains) and test it against proteins C, E and H (all lambda chains), with the following results:

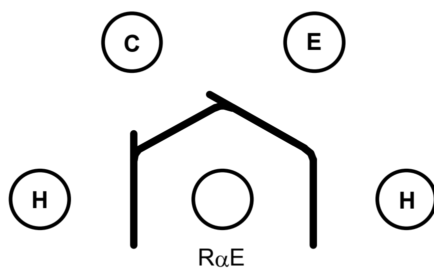


Figure 6-4

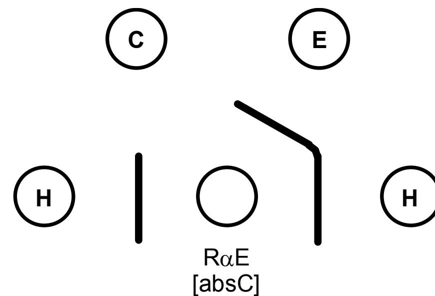


Figure 6-5

On the left is the pattern shown by the original antiserum, and on the right the pattern seen after the antiserum has been absorbed with protein C, as we had done before with the anti-kappa antiserum. We see that not all the lambda proteins are the same, *i.e.*, proteins E and H have at least one epitope that protein C lacks. We have thus defined a variant of human lambda chains which we call *Oz*; proteins E and H are Oz^+ while protein C is Oz^-.

Once again we ask if this represents an *allelic* variant of lambda chains, as Km is for kappa chains. When we test a large panel of normal human sera, we find that *every* serum contains Oz^+ lambda chains. (Every serum also contains Oz^- lambda chains, which we can determine by further analysis). Therefore we are not dealing with an allotype, but a *new isotypic variant*. While our earlier analysis had indicated the existence of at least two genetic loci for human light chains, one for kappa and one for lambda, we now have to hypothesize at least *two distinct loci* for lambda chains, one for each the two variants we have just defined (Oz^+ and Oz^-).

Since the original discovery of the Oz marker, two other isotypic serological markers have been found for human lambda chains, namely *Mcg* and *Kern*. These three specificities are found in four different combinations which define a total of four genetic loci encoding human lambda chains, named *lambda-1* through *lambda-4*. No *allelic* variants have yet been described for any of these four lambda isotypes, however.

EXPERIMENTAL DEFINITION OF IDIOTYPES:
INDIVIDUALLY SPECIFIC EPITOPES

We will now examine the results of one additional set of analyses, this time following immunization of a rabbit not with a purified light chain, but with an entire *intact myeloma protein*. If we start (for example) with a protein which has a kappa light chain and a gamma-1 heavy chain (*i.e.,* a κG1 protein) we can predict the presence of specific antibodies to the *four distinct varieties of known epitopes* in the resultant antiserum:

anti-kappa. The rabbit should make antibodies which react with *all* kappa chains (anti-isotype), just as it did when we immunized with purified kappa chain.

anti-G1. We also expect to find antibodies which will react with all G1 heavy chains, defining the G1 isotype.

anti-Km. We might also expect to have anti-allotype antibodies. If the kappa chain happened to be Km(1), for instance, the rabbit should produce anti-Km(1), equivalent to the antibody we originally called "anti-Inv" in the example above.

anti-G1m. Similarly, we might expect the rabbit to recognize whatever G1m allotypic markers are present on the heavy chain. If the heavy chain happened to be G1m(17), for example, the rabbit could make antibodies to this epitope. (NOTE: While we need to consider this possibility, anti-Gm antibodies are not easily produced by heterologous immunizations, as in this example.)

Each of these categories of antibodies can be detected by Ouchterlony precipitation using an appropriate variety of myeloma proteins as antigens. Each of these four represents a *separate and distinct population of antibodies*, and each can be *removed* by appropriate absorption (as we have described above). What happens when we absorb (remove) all antibody activity to the four kinds of specificities listed above, then test the resultant absorbed antiserum against the original κG1 immunogen (as well as a variety of other myeloma proteins)? *Our prediction is that there should be no detectable remaining antibody, since we have removed antibodies to all those epitopes we know about.*

However, what we actually find is that **the absorbed antiserum still reacts with the original κG1**, although it does *not* react with any other myeloma protein (of *any* isotype or allotype composition). This antibody, therefore, defines an *idiotype*, or idiotypic epitope/specificity. It is "individually specific", reacting with the original immunogen but not with any other. Nor will it react with isolated light or heavy chains, even from the same protein, but it requires the presence of the *intact molecule*. Such an antibody is binding to an epitope created by the *combination of a particular* V_H *and* V_L *domain*, that is, *the unique antigen-combining site* of the immunizing myeloma protein. This idiotype will in general not be detectable in normal sera; even though the particular combination of V_H and V_L *may* be present on some normal serum Ig molecules, they will only be a few molecules among many thousands of different combining sites present in normal Ig and would be extremely difficult to detect.

CHAPTER 6, STUDY QUESTIONS:

1. Define and distinguish antibody ISOTYPE, ALLOTYPE and IDIOTYPE.

2. Given a newly produced antiserum directed against a human Bence-Jones protein, how would you determine if it recognized ISOTYPE or ALLOTYPE specificities?

3. How might you produce an *anti-G3*-specific antiserum? An *anti-Km(2)*-specific antiserum?

4. How would you produce an anti-IDIOTYPE antiserum?

CHAPTER 7
CELLULAR BASIS OF ANTIBODY DIVERSITY: CLONAL SELECTION

The *specificity* of humoral immune responses relies on the huge DIVERSITY of antigen combining sites present in antibodies, *diversity which is generated in an antigen-independent fashion*. The process by which antigen (during an immune response) stimulates the clonal expansion and differentiation of antibody-forming cells of predetermined specificity is known as CLONAL SELECTION.

The basic principles of clonal selection are illustrated in this chapter by two classic experiments, and are applied to our understanding of some of the fundamental features of the adaptive immune response, namely TOLERANCE, MEMORY and AFFINITY MATURATION, as well as the presence of NATURAL ANTIBODIES.

A humoral immune response typically generates antibodies with a wide diversity of combining sites; even a simple hapten will elicit a highly heterogeneous population of antibodies. In order to provide a framework for understanding how the diversity of the antibody response is generated, we can set out three facts regarding antibody specificity which are based on experimental findings:

1) **An animal can produce antibodies to many different epitopes**. This has been known since the early days of immunology, based simply on the variety of distinct molecules that antibodies can distinguish. How many antibody combining sites exist in nature? For a start let's say there are at least one million different combining sites (although this is certainly a gross underestimate).

2) **A single antibody-secreting cell produces only one kind of antibody**. All molecules of antibody produced by a single plasma cell are identical with respect to isotype, allotype and idiotype specificities. The heterogeneity of antibody responses, therefore, reflects the heterogeneity of antibody-forming cells, and *not* that of the products of each individual cell. This explains the *homogeneity* of myeloma proteins, since they are the products of clonally derived tumor cells. (NOTE: One important consequence of this fact is **allelic exclusion**, which will be discussed later [see Chapter 9].)

3) **The specificity of antibodies is determined by the primary structure (*i.e.,* the amino acid sequence) of the light and heavy chain variable regions**. Antibody diversity *cannot* be explained by different folding patterns of molecules with identical amino acid sequences (as had erroneously been proposed in the 1940's).

Let's examine the problem of the generation of immune responses at the cellular level. How does a particular antibody-forming cell "know" which of the million possible combining sites it is "supposed" to produce? Two general kinds of schemes can be invoked to explain this, namely those of **instruction** and **selection**. These two schemes differ in the assumptions they make about the hypothetical *precursors* of the antibody-forming cells (*"AFCP"s*).

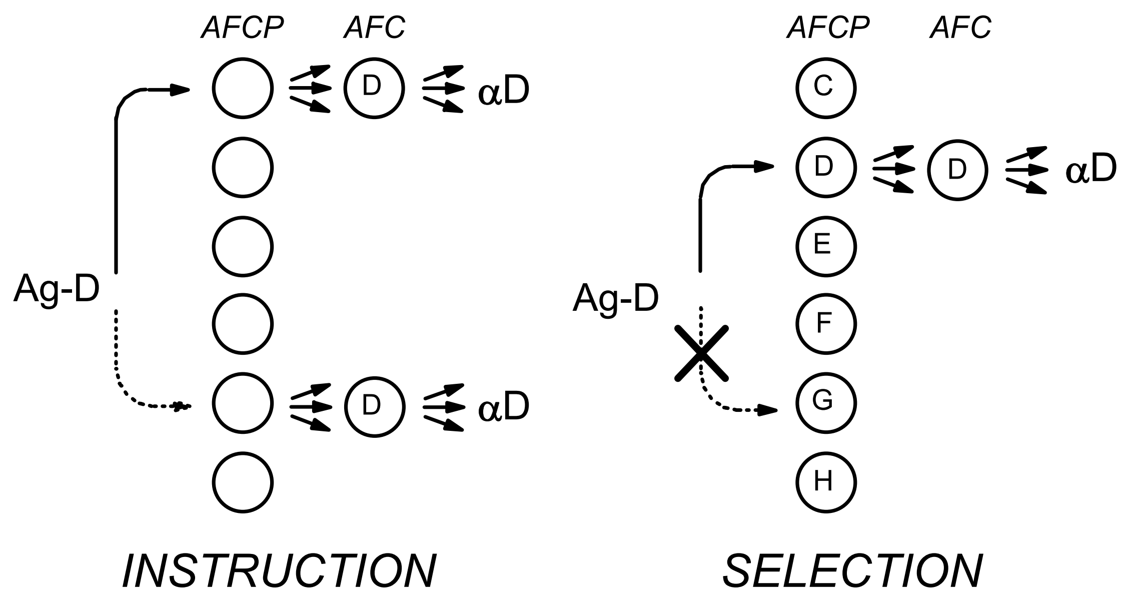


Figure 7-1

Under INSTRUCTIONAL schemes, the precursor of an antibody-forming cell is *not* precommitted, but has the potential of making any one of a million different antibodies. This multipotential precursor cell, following exposure to antigen, somehow recognizes which of the million possible antibodies is needed and proceeds to differentiate into an antibody-forming cell producing that particular antibody; proliferation also takes place, expanding the number of antibody-forming cells over the number of precursors. Under such a scheme *every* precursor cell can potentially respond to *any* antigen.

SELECTIONAL schemes, however, hypothesize that the precursors of antibody-forming cells are themselves *precommitted* to producing antibody of a particular specificity. Only a small fraction of the precursor cells is capable of responding to any particular antigen. The consequences of antigen exposure are the same as described for the instructional scheme, *i.e.* differentiation into mature antibody-forming cells, and multiple rounds of proliferation which greatly expand the number of antibody-forming cells.

CLONAL SELECTION

One formulation of the selectional class of schemes is known as CLONAL SELECTION, and was developed in the 1950's by Burnet based on work of Jerne and Talmadge. *This hypothesis has become the foundation of modern immunology*, and states the following:

1) Each Antibody-Forming Cell Precursors (AFCP) is ***precommitted*** to making antibody of a particular specificity, ***even before it ever encounters a target antigen***.

2) Each AFCP bears ***membrane-bound immunoglobu*lin** displaying the combining site it is capable of making, in effect, a "sample of its wares".

3) This membrane Ig acts as a ***specific receptor molecule*** for the particular epitope to which it is directed. Upon binding to an AFCP's surface Ig, the antigen stimulates that cell to differentiate into an antibody-forming cell, and to proliferate (**clonal expansion**).

Two central features of Burnet's Clonal Selection Hypothesis should be emphasized. First, it makes the simplifying assumption that **the only molecule that can specifically recognize an epitope is antibody** itself. By hypothesizing the presence of antibody pre-existing on the surface of the AFCP, Burnet avoided the problem of how a non-committed precursor cell "looks" at antigen to determine which of the many possible antibodies is needed. Second, it states that **the development of the immunological repertoire is antigen-independent**. The *differentiation* which results in the appearance of a particular AFCP must be a developmentally controlled process, and must be able to take place *in the absence of any antigen stimulation*.

EXPERIMENTAL CONFIRMATION OF CLONAL SELECTION

Let's examine two key experiments, carried out in the late 1960's, which provided compelling evidence for the idea that AFCPs are precommitted to a particular antigen. In one case specific AFCPs are killed as a result of binding a specific radioactive antigen, in the other the AFCPs are killed as a result of their proliferative response to their particular antigen.

Hot Antigen Suicide. Ada and Byrt (1969) reasoned that if AFCPs bear surface receptors which can specifically bind antigen, then these cells should be susceptible to being killed if presented with a toxic form of antigen. They used an ADOPTIVE TRANSFER system, consisting of injecting normal spleen cells together with antigen into a lethally irradiated recipient. Testing the recipient's spleen for the presence of antibody-forming cells showed a high response at the end of seven days.

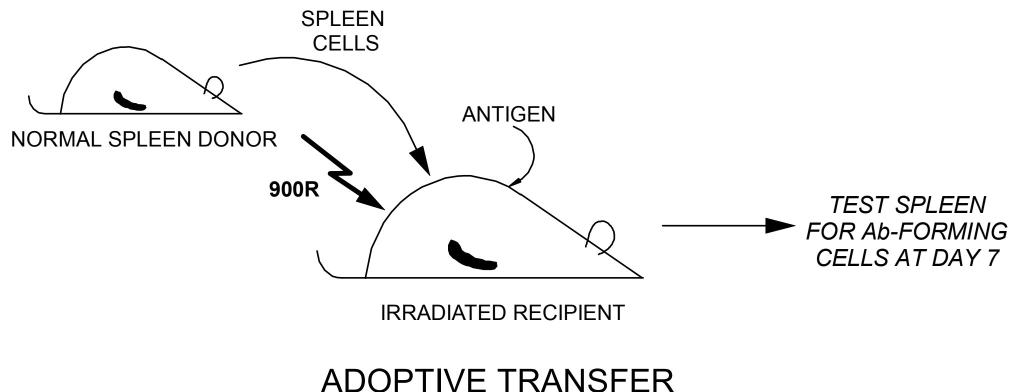


Figure 7-2

They then took a sample of the antigen (Salmonella flagellin, or "Fla") and made it highly radioactive by coupling it with Iodine-125. This radioactive antigen was incubated overnight with the normal spleen cells *before* transfer into the irradiated recipient. The rationale is that if precursor cells are *precommitted* to a particular antigen, and if they have *cell surface receptors* specific for the antigen, then those cells precommitted to the antigen Fla, and *only* those cells, should *bind the radioactive antigen* and thus commit "suicide" due to the radioactivity.

The results they obtained are summarized below:

	Ag used for preincubation	Ag transferred with cells	Resulting 7-day Ab response
1)	none	none	none
2)	none	Fla	high
3)	^{125}I-Fla	Fla	low
4)	^{125}I-Fla	Fla + BGG	low anti-Fla high anti-BGG

Spleen cells incubated with "hot" Fla can no longer transfer responsiveness to Fla, although their response to an unrelated antigen is (BGG) unaffected.

These results show that the precursors of antibody-forming cells for the anti-Fla response can be eliminated by incubating the spleen cells with highly radioactive Fla, but that this treatment does *not* affect the precursors for the anti-BGG response. *The precursors of anti-Fla antibody-forming cells are a different population from the anti-BGG precursors, and this precommitment is reflected in their ability to specifically bind the antigen to their surface*. These are the two key elements of Burnet's Clonal Selection Theory.

^{3}H-TdR Suicide. Mishell and Dutton (1966) took a slightly different approach when they developed a system in which they could generate an *in vitro* primary antibody response. Under a carefully specified set of culture conditions, they incubated normal mouse spleen cells together with antigen, and after five days they were able to demonstrate the presence of large numbers of antibody-forming cells (PFC's, for Plaque-Forming Cells) in the culture dish (line 1 in Figure 7-3).

They then used highly radioactive tritium-labeled thymidine (^{3}H-TdR) to selectively kill those cells undergoing proliferation. (Thymidine is a DNA precursor which is taken up and incorporated only by those cells synthesizing DNA, *i.e*., proliferating cells; "resting" cells are not affected by this treatment.) By treating with "hot" thymidine during different 24-hour periods, they were able to show that the precursors of antibody forming cells were proliferating (and therefore could be killed by ^{3}H-TdR) between 24 and 48 hours after initiation of the culture with the antigen SRBC (sheep red blood cells; see line 3); treating the cells during the *first* 24 hours after initiation had no affect on the resulting response (seen in line 2).

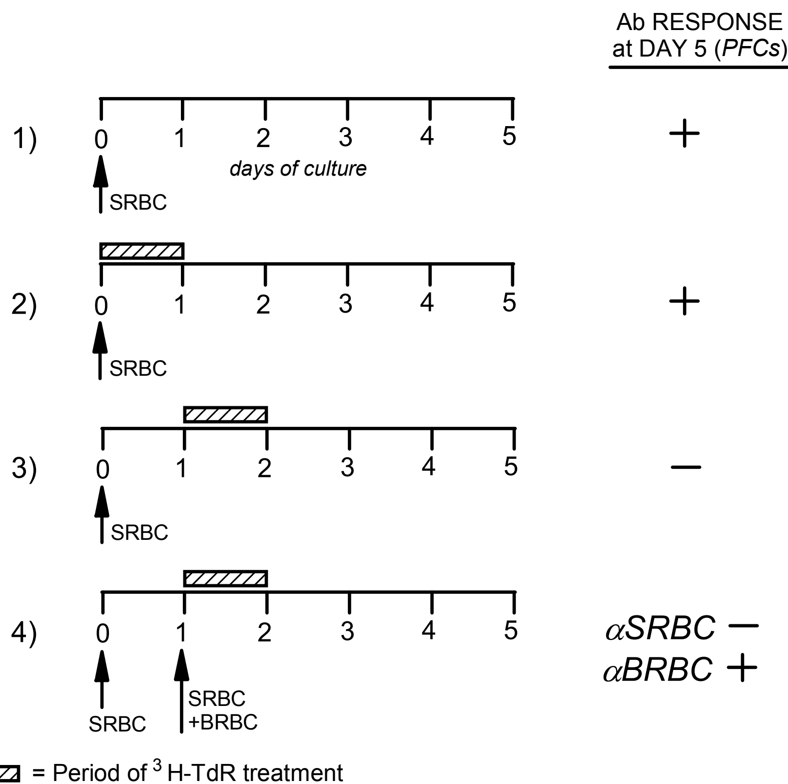


Figure 7-3

The key part of the experiment is in line 4. A ^{3}H-TdR pulse between 24 and 48 hours should eliminate the response to the original SRBC challenge (as in line 3). However, if AFCPs are not precommitted, then it should *not* affect the response to a second dose of SRBC given at 24 hr (see line 2), nor should it effect the response to a different antigen (BRBC, burro red blood cell) given at the same time. But what they found was that while the response to BRBC was high, as expected, the response to the second dose of SRBC was very low.

These results show that the population of precursor cells which proliferate in a *specific response* to antigenic challenge are *different* for the closely related antigens SRBC and BRBC (burro red blood cells). Following the "hot" pulse at 24-48 hr, which kills all those precursors which had responded to SRBC, there is *no other* remaining population of precursors which can respond to SRBC (as there would be under an "instructional" scheme), although the precursors for a different antigen have not been effected by this treatment.

These two sets of experiments, and many more in later years, confirmed the basic features of CLONAL SELECTION--*separate precommitted populations of precursor cells respond (by proliferation and differentiation) to different antigens, and each have antigen-specific receptors on their surface.*

CLONAL SELECTION AND TOLERANCE

One of the most striking features of Burnet's Clonal Selection theory was its simple explanation of the phenomenon of SELF-TOLERANCE, an organism's normal lack of ability to make immune responses against its own "self" components, while simultaneously being able to respond to any "foreign" antigen Burnet hypothesized that during the development of an antibody-forming cell precursor, it goes through a *short-lived stage in which exposure to its specific antigen will result in its own death*. This would ensure that no precursor can develop which recognizes antigens ordinarily present in the organism, but all other precursors would develop normally. This represents tolerance by the mechanism of CLONAL ABORTION, and was the most attractive explanation of the phenomenon of tolerance for many years.

The phenomenon of tolerance will be discussed at greater length in Chapter 18, and we will find that while clonal abortion is known to take place and to play an important role, it clearly fails to account for some of the features of natural or experimental tolerance. It can be shown in many cases, for instance, that some clonally specific precursor cells specific for "self" antigens *do* exist in normal animals; therefore, the fact that they do *not* ordinarily respond and become antibody-forming cells must be explained on some basis *other* than clonal abortion.

CLONAL SELECTION AND MEMORY

Antigen-dependent proliferation of clonally precommitted cells has at least two important consequences.

1) Production of a large number of effector cells of that particular specificity (**Clonal Expansion**). A single antigen-reactive precursor may give rise to hundreds of thousands of antibody-forming cells.

2) Production of an expanded number of **memory cells**. These memory cells are themselves antigen-reactive cells, which can respond to antigen stimulation by proliferation and differentiation into antibody-forming cells. However, they differ from the original "virgin" precursors in several ways, including that they are considerably more numerous., and that they respond much more rapidly to antigenic stimulation,

The increased speed of response of memory cells, as well as their higher numbers, is the basis of immunological memory. This will be discussed in more detail in Chapter 9 (see Figure 9-3).

CLONAL SELECTION AND AFFINITY MATURATION

We have already noted that secondary (memory) responses produce antibody of higher affinity than primary responses. This is one manifestation of the phenomenon of **affinity maturation** which can also be readily understood in the context of clonal selection. The heterogeneity of antibody responses is a reflection of the heterogeneity of AFCPs. Many *different* precursors will exist, for example, which can recognize the hapten DNP, each with its own characteristic affinity. Let's assume, for the argument, the existence of seven different precursor cells with affinities for DNP ranging from 10^4 to 10^{10}. In a typical

immune response there will be enough antigen to trigger all seven (they will all differentiate and proliferate), resulting in an average antibody affinity of about 10^7 (see the left hand side of Figure 7-4).

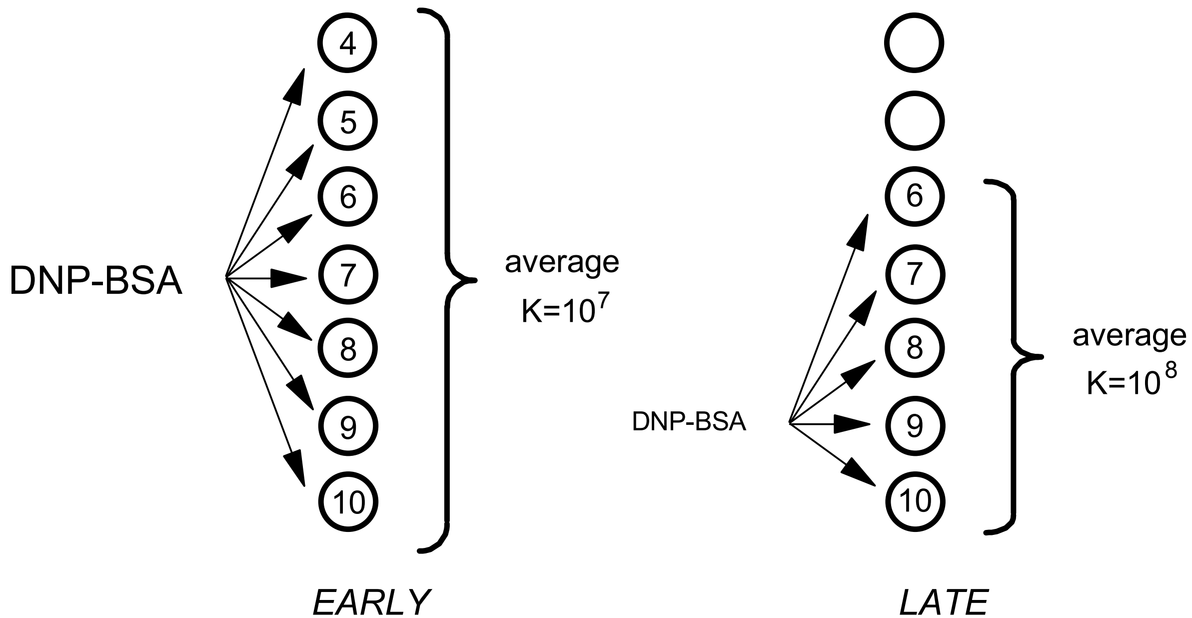


Figure 7-4

As the response progresses, one of its consequences is the elimination of antigen (by "processing", opsonization and phagocytosis, *etc.*). Before the antigen is completely eliminated, however, the small amount that still remains may continue to trigger available precursor cells. However, *if the amount of remaining antigen is limiting* and there is not enough to trigger the entire population of available precursors, *those precursors for higher affinity antibody will be selectively triggered*. If, in the example above, only those precursors in the range of 10^6 to 10^{10} are re-stimulated later in the response, they will be selectively expanded and the average affinity of serum antibody will increase toward 10^8 (shown on the right-hand side of Figure 7-4).

Thus, affinity maturation is a logical consequence of the heterogeneity of precommitted AFCPs, and of the fact that antigen can continue to trigger precursor cells as long as it is present. (The process of "somatic hypermutation", discussed in Chapter 9, increases the diversity of antigen-combining sites during immune responses, thus contributing to even more effective affinity maturation.)

"NATURAL" ANTIBODIES?

As we have seen, antibodies are produced following stimulation by specific antigens. The total immunoglobulin in our serum, which typically amounts to 10-15 mg/ml (1-1.5 gm/dl), is the result of extensive exposure to many immunogenic substances in our food and on the

microorganisms which inhabit and infect us, as well as from deliberate vaccinations. Experimental animals which are raised under germ-free and antigen-free conditions have little or no detectable serum Ig; if they are subsequently exposed to a conventional environment, however, their Ig levels rapidly rise to reach the normal range.

Are there any antibodies which can truly be called "natural" and which are not the result of specific antigen stimulation? Perhaps, but only in a limited sense. It is important to recognize that immune responses often include *non-specific* components -- in addition to resulting in a specific antibody response, antigenic stimulation may result in non-specific stimulation of nearby "bystander" B-cells, a phenomenon known as POLYCLONAL ACTIVATION (we will discuss some of the mechanisms involved later in Chapters 12 and 15.) This is thought to reflect the non-specific stimulation of pre-existing memory cells (among others) and, therefore, will tend to be biased toward the products of previous immune responses. In general, the term "natural antibody" simply means antibody which is not the result of a specific and *known* antigenic stimulation.

CHAPTER 7, STUDY QUESTIONS:

1. How does CLONAL SELECTION differ from *instructional* theories in explaining antibody diversity?
2. How do the experiments of Ada and Byrt (*hot antigen* suicide) and Mishell and Dutton (*hot thymidine* suicide) support Clonal Selection? How do they differ from one another?
3. Does either of the above experiments *exclude* the possibility that a single AFCP may be capable of producing *two* different antibodies with different specificities? Does this situation occur in nature?
4. Why is affinity maturation a logical consequence of Clonal Selection?

CHAPTER 8
GENETIC BASIS OF ANTIBODY DIVERSITY

SEE APPENDIX (9) SOUTHERN BLOTTING

Underlying the development of antibody diversity is a unique pattern of gene organization and molecular events. THREE FAMILIES OF IMMUNOGLOBULIN GENES exist in mammals, one encoding HEAVY chains, another KAPPA chains, and the third LAMBDA chains. Each of these clusters contains one or more *constant region* genes and a number of *variable region* gene segments. The formation of a complete variable region of a light or heavy chain requires the joining of two or three separate genetic elements by a process of GENE REARRANGEMENT; a separate DNA rearrangement in the heavy-chain complex is required for subsequent CLASS-SWITCHING. Both germ-line and somatic events contribute to antibody diversity, including COMBINATORIAL JOINING, SOMATIC MUTATION and COMBINATORIAL ASSOCIATION. This gene organization and the requirement for gene rearrangement is unique to immunoglobulins, with the single exception of the related family of genes encoding the T-CELL RECEPTORS, whose functions will be discussed later.

The problem of the genetic basis of antibody diversity is one which was hotly debated through the 1960's and 70's, and only through the application of recombinant DNA technology have its essential features become well understood.

The basic problem can be set out as follows: if there are one million different antibodies which the immune system can produce, and if each of them has a unique primary structure (*i.e.*, amino acid sequence), are there then a million genes required for their production, or can a smaller number of genes be modified in some systematic way to account for the total diversity? These two possibilities define the essential features of two competing hypotheses, GERM LINE generation of diversity on the one hand *versus* SOMATIC generation of diversity on the other.

We can simplify the problem by recognizing that the antibody combining site (which is, of course, responsible for specificity) is made up of two elements, the V_H and V_L. If as few as one thousand V_L domains combine randomly with the same number of V_H domains, we could account for one million different combining sites ($10^3X10^3=10^6$).

If we use the kappa light chain system as an example (recognizing that the same arguments can be applied to the other V-region families), we can define the extreme forms of GERM LINE and SOMATIC theories as follows:

GERM-LINE THEORY -- For every kappa-chain V-region there exists one *unique* germ-line gene. A particular antibody-forming cell selects one of these and expresses it in unmodified form.

SOMATIC THEORY -- Only a *single* germ-line gene exists for *all* kappa-chain V-regions. A particular antibody-forming cell expresses this gene following a process of somatic mutation, which results in each cell expressing a different version of this gene.

Decades of genetic studies, culminating in the cloning and sequencing of immunoglobulin genes, have shown that *significant features of both theories are correct*. ***Many genes exist for V-regions*** of immunoglobulins, and ***these genes are somatically modified*** in a variety of ways in the course of their expression in antibody-forming cells.

STRUCTURE AND EXPRESSION OF IMMUNOGLOBULIN GENES

Three families of immunoglobulin genes exist, each on a separate chromosome. One includes *kappa* genes, another *lambda* genes, and the third includes all the *heavy chain* genes. Each family consists of a *series of V-regions* genetically linked (but still separated by long stretches of DNA) to *one or more C-regions*.

We can diagram the three *human Ig* clusters as follows:

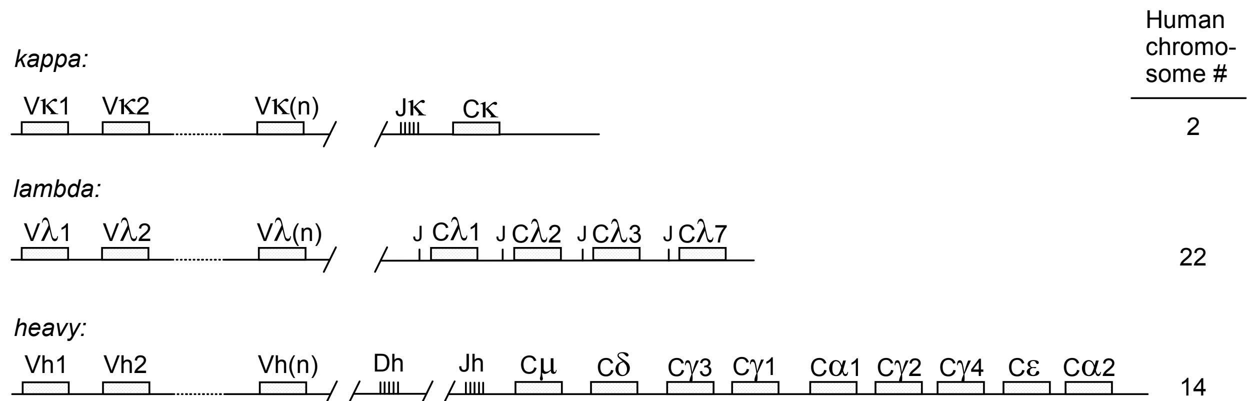


Figure 8-1

Let's examine in some detail the structure of the *kappa chain complex* and the processes involved in its expression, referring to Fig. 8-2 below. This complex consists of a large number of V-region genes (about 55) genetically linked (by a long stretch of DNA) to a single copy of the constant region gene. An additional cluster of five short gene segments called J-segments is located a few thousand base pairs upstream (5' direction) of the C-region gene, and each of these codes for the last 13 amino acids of the variable region. (*NOTE: Don't confuse these "J-segments" with the "J-chain," the polypeptide attached to IgM and polymeric IgA, which we will discuss in Chapter 9*.) This is the "germ-line" configuration, present in germ cells (sperm and eggs) as well as all somatic cells other than Ig-producing cells, and is shown at the top of Figure 8-2.

The first step in expression of this gene is DNA REARRANGEMENT, involving the joining of one V-region and one J-segment, each chosen at random in any given B-cell. The result is the structure in line 2 of the figure, and the DNA which was originally *between* the selected V and J genes is *cut out and lost* in the form of a closed circular molecule; other V-region

MOLECULAR BASIS OF KAPPA GENE EXPRESSION

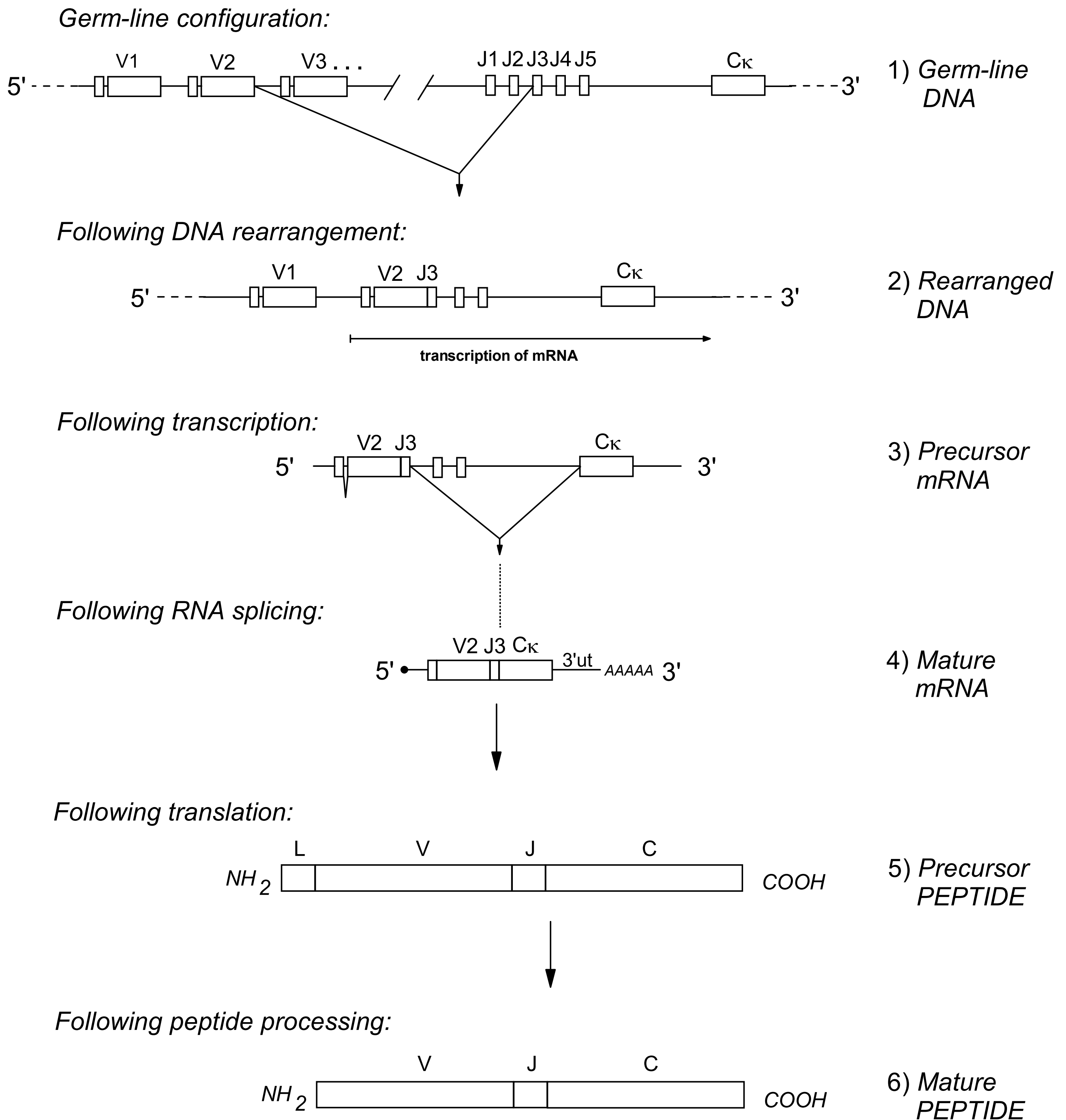


Figure 8-2

gene segments which happen to reside outside this excised segment (to the left, or 5', of the V-region which is used (for example, V1 in line 2 of Figure 8-2) are retained, although they are no longer relevant to expression, described below. *This process is unique to immunoglobulin (and T-cell receptor) genes*.

The process of TRANSCRIPTION starts at the beginning of the rearranged V-region and continues past the end of the C-region, resulting in an immature mRNA whose structure is shown in line 3. The large intervening sequence ("intron") between the J-segments and the C-region is removed by the process of RNA SPLICING, resulting in the mature mRNA shown in line 4. It includes a 200 nucleotide region at its 3' end which is not translated, and a 3' poly-A "tail". *The structure of this transcript and the processes by which it was produced (transcription and RNA splicing) are characteristic of most eukaryotic genes*.

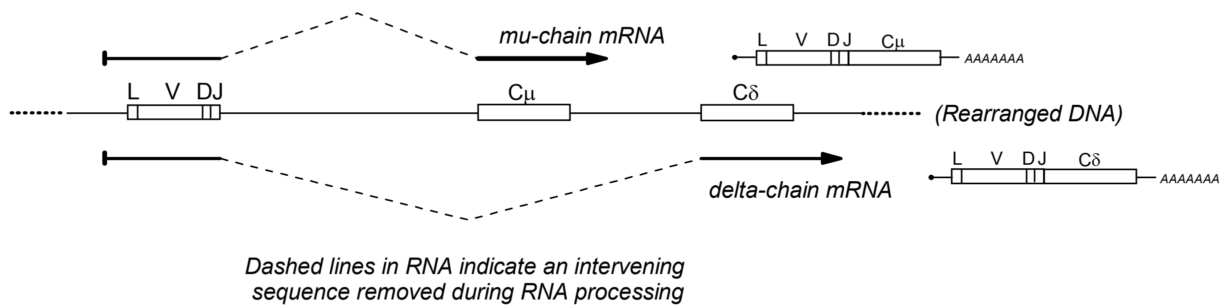
TRANSLATION of this message occurs on ribosomes associated with the rough endoplasmic reticulum (RER), and the resulting polypeptide has the structure shown in line 5. It is identical to the known structure of immunoglobulin kappa chains with a single exception -- it has an additional 13 or so amino acids at its amino terminal end, known as the LEADER or SIGNAL sequence. This sequence is required for the transport of the polypeptide across the endoplasmic reticulum membrane into the lumen of the ER, and is *cleaved off* when the polypeptide moves into the cisterna of the rough ER. *The presence of the leader sequence and its proteolytic cleavage are general features of all secreted proteins in eukaryotes and prokaryotes*.

The structure and mechanism of expression of *lambda chains* and *heavy chains* are similar to what we have just described for kappa chains--all have J-SEGMENTS, all show DNA REARRANGEMENT, TRANSCRIPTION, RNA SPLICING, TRANSLATION and proteolytic cleavage of the LEADER POLYPEPTIDE. Heavy chain gene structure is somewhat more complex, however, as there also exists an additional cluster of gene segments (known as "D", for "diversity", segments) which each encodes four amino acids between the V-region cluster and the J-segments. DNA rearrangement for H-chains thus involves *two* events, joining of a V with a D, and joining of the D with a J-segment. Transcription and the other processes discussed above take place as they do for kappa genes.

In each case, the end result is a polypeptide whose amino acid sequence has been determined by three or four separate genetic elements, and which is incorporated into the final immunoglobulin molecule by processes which will be discussed in Chapter 9.

ALTERNATE SPLICING IN B-CELLS

One unusual phenomenon still needs to be explained, *i.e*., the simultaneous synthesis of IgM and IgD by a single B-cell. *This is the only example of a normal cell simultaneously producing two different kinds of immunoglobulin*. The explanation derives from the fact that mu (μ) and delta (δ) constant region genes are adjacent to one another in the heavy chain gene complex, as illustrated in Figure 8-3. Using the same rearranged heavy chain V/D/J complex, a B-cell can make two kinds of mRNA--it can transcribe from the V-region through the end of the Cμ gene and make IgM, or it can transcribe all the way through the Cδ gene and make IgD *by splicing out the Cμ region together with the intervening sequence during RNA splicing*. Two different mRNAs can thus be made from a single gene complex. It should be emphasized that alternate splicing of RNA is a mechanism used by many other genes to generate diverse protein products.



SIMULTANEOUS SYNTHESIS OF IgM AND IgD IN B-CELLS BY ALTERNATE RNA SPLICING

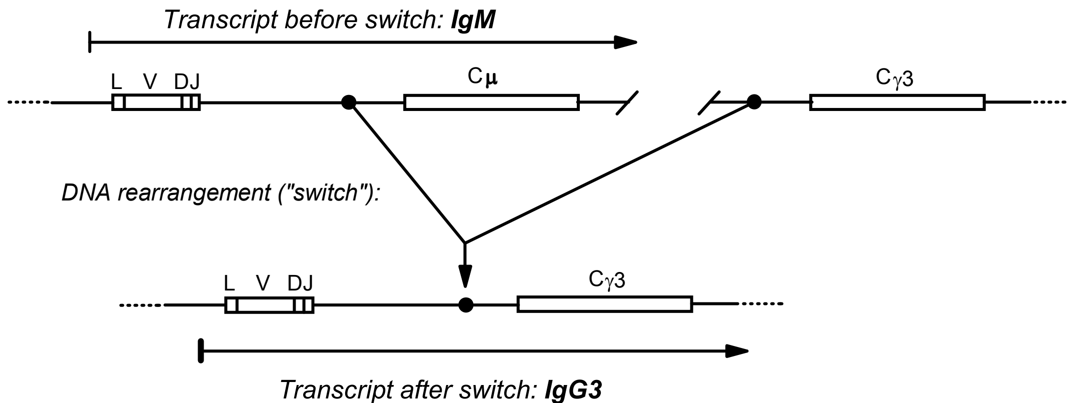
Figure 8-3

This process accounts for two important phenomena. *First*, IgM and IgD synthesis in B-cells can occur *simultaneously and continuously* (it is *not* a transient result of a switch from mu to delta). And *second*, it has been shown that the *mu and delta chains are produced from the same chromosome*, and not from the two different allelic copies. This is an extension of *allelic exclusion* which will be discussed in Chapter 9, and is known as *haplotype exclusion*.

Alternate splicing of mRNA also accounts for another important feature of immunoglobulin expression, namely the choice of whether a *secreted versus a membrane-bound form* of Ig is produced. All classes of Ig can be produced either as membrane-bound molecules expressed on the surface of B-cells, or as secreted molecules released into the serum or extracellular space. This difference is determined by which of two alternate exons is selected to be present at the 3' end of the heavy chain mRNA, which in turn depends on alternate mRNA splicing. Regulation of such mRNA splicing is therefore an important element in the differentiation of the B-cell lineage.

HEAVY CHAIN CLASS SWITCHING: A SECOND DNA REARRANGEMENT

While antibody-secreting cells begin their existence by secreting IgM, the progeny of an individual cell can go on to produce IgG, IgE or IgA. This phenomenon is known as CLASS SWITCHING and occurs by *another DNA rearrangement* event (shown in Figure 8-4, below), which relocates the already rearranged V/D/J complex from its original position near the Cμ gene to a position close to one of the other heavy-chain C-regions (γ3 in this example). This results in a new transcription unit and the synthesis of *a heavy chain with same V-region but a new C-region*.



MOLECULAR BASIS OF CLASS SWITCHING

Figure 8-4

This process accounts for two of the key features of class switching. First, as mentioned above, *the V_H-region remains the same*. Since switching does not change the light chains, this means that the specificity of the antibody (and its idiotype) cannot change. Second, *class switching is unidirectional and irreversible*. A cell can switch from one C-region to another situated to the right of the first (5'-->3' direction), but cannot go backwards (3'-->5' direction). The order of switching is therefore defined by the order of the heavy chain constant region genes on the chromosome. *[NOTE: C_H genes are each made up of a number of exons and introns which are not shown in this schematic.]*

SOURCES OF ANTIBODY DIVERSITY: SUMMARY

Let's return to our original question of the Generation of Diversity and examine the genetic basis for the existence of a million or more antibody specificities. We'll use what we now know of human *kappa chain* gene structure and expression as our primary example.

1) **About 50 Vκ-genes exist in the human genome**. This is a fairly large number, much higher than somatic theories predicted, although smaller than predicted by most germ-line theorists.
2) **Five different Jκ-segments exist in the human genome**. Thus, if every V-region can be used with any J-segment, a total of about 250 (50 x 5) different combinations can be made. (In the case of heavy chains, the existence of several D-segments increases this number even more.) This process is referred to as COMBINATORIAL JOINING.
3) **DNA rearrangement is imprecise**. The joining of V and J (and the joining of D segments to V and J) is a deliberately *error-prone process*, resulting in random nucleotide substitutions and deletion/insertions at the site of rearrangement. Therefore, the joining of a particular V-region and a particular J-segment can yield *different* results in different cells in which it occurs. If this increases the number of potential V-regions by about ten-fold, we now have about 2500 possible kappa chain V-regions (250 x 10).
4) **Somatic mutation occurs in rearranged V-regions**. Expressed kappa chain genes have been cloned and sequenced from many antibody secreting cells, and in most cases

they have been found to differ from *any* existing germ-line V-region in sequence positions *other* than the site of joining of V and J. These somatic mutations tend to be localized to the hypervariable regions, and occur by a local relaxation of the normal processes of error-correction in newly synthesized DNA. The presence of these mutations may increase the number of possible V-regions by another ten-fold or more, resulting in at least 25,000 different kappa V-region sequences (2500 x 10).

5) **Heavy and light chains associate in random combinations**. If a comparable number of different H-chain V-regions can be produced (which is an underestimate), then there are potentially some 6×10^8 different antibody combining sites (25,000 X 25,000), a number considerably larger than the one million we initially set out to explain. This random association of H- and L-chains is referred to as COMBINATORIAL ASSOCIATION.

It has therefore become clear that the historical debate between proponents of GERM-LINE and SOMATIC theories of antibody diversity is no longer meaningful, and that elements of *both* theories are important in the generation of antibody diversity. Points 1, 2, and 5 above fall within the framework of the *germ-line* theory, while points 3 and 4 quite clearly represent *somatic* processes.

T-CELL RECEPTORS: Ig-LIKE GENE ORGANIZATION AND GENE REARRANGEMENTS

The organization of immunoglobulin V- and C-region segments, and the processes by which they are rearranged, are clearly well-adapted for the generation of the huge diversity which so important for the humoral immune system. Such gene rearrangements are, in fact, unique to the immunoglobulin genes, with the single exception of the T-cell receptor (TCR), whose roles in immune responses we will discuss later (see Chapters 14, 15 and 18). These molecules, although quite distinct from immunoglobulins, are evolutionarily related to them, and share many important properties with them. They are a highly *diverse* family of *heterodimeric membrane receptors* capable of specifically interacting with antigens, their polypeptide chains include *variable and constant regions*, and the genes encoding them are *organized and rearranged* in a manner very similar to those of immunoglobulins.

There are, however, three critical differences in the structure and expression of TCR's compared with Ig: 1) Each TCR molecule bears only a *single* combining site (it does not exhibit the bivalency characteristic of *all* antibody molecules); 2) Somatic mutation, an important component in the generation of antibody diversity, does *not* occur during TCR expression. 3) Unlike immunoglobulins which can exist as either soluble or membrane-bound forms, TCR's function *only* as membrane-bound molecules. In later chapters we'll discuss the unique biology and antigen-recognition functions of the TCR, and it will become clear why these differences between Ig and TCR are centrally important.

RAG-1 AND RAG-2 RECOMBINASE

Despite the important differences between immunoglobulin and T-cell antigen receptors, they both express unique antigen-combining sites which, as described above, can be assembled only as a result of V(D)J recombination of germ-line DNA. In both cases this process requires the participation of two proteins known as *RAG-1* and *RAG-2* (for "***R***ecombination ***A***ctivating ***G***enes"). These recombinase enzymes first appeared during the evolution of cartilagenous fish, and are present *only* in those organisms capable of carrying out *adaptive immune responses*, namely the "higher" vertebrates, including fish, reptiles, birds, amphibians and mammals. *Genetic knock-out of the RAG proteins in mice results in the complete inability to produce either immunoglobulins or T-cell receptors*, and consequently the absence of both mature B-cells and T-cells. Such knock-outs have been widely used to manipulate and study the vertebrate immune system.

CHAPTER 8, STUDY QUESTIONS:

1. Describe the overall architecture of the three mammalian IMMUNOGLOBULIN GENE FAMILIES.
2. Describe the molecular processes involved in expression of a kappa light chain gene. What processes are unique to immunoglobulins? In what ways does expression of a *heavy* chain differ from that of light chains?
3. What are the various processes which contribute to antibody diversity?
4. In what ways can DNA rearrangements be used to help define and diagnose lymphocyte tumors?
5. What would be the expected phenotypic consequence of a *null* mutation in the human RAG-1 or RAG-2 gene?

CHAPTER 9
IMMUNOGLOBULIN BIOSYNTHESIS

Although the process by which a functional gene for immunoglobulin HEAVY and LIGHT CHAINS is formed is highly unusual, the SYNTHESIS, POST-TRANSLATIONAL PROCESSING and SECRETION of these glycoproteins all occur by conventional pathways. The J-CHAIN is required for polymeric Ig and the SECRETORY PIECE for the unique process by which secretory IgA is transported into exocrine fluids. Knowing the molecular and cellular mechanisms involved in Ig production, we can understand the importance of ALLELIC EXCLUSION and the structural differences between SECRETED *versus* MEMBRANE-BOUND FORMS of Ig, and provide a rational basis for the existence of CLASS SWITCHING.

Most circulating immunoglobulin is produced by plasma cells, which are highly specialized and efficient antibody "factories". The biosynthesis of immunoglobulins has much in common with the biosynthesis of other secreted proteins. Several aspects of this process, however, are unusual, and some features are quite unique to immunoglobulins.

The structural components of Ig molecules we need to account for are as follows:

- Light Chains
- Heavy Chains
- Carbohydrate (on H-chain only)
- J-Chain (attached to H-chain of IgM and polymeric IgA)
- Secretory piece (attached to H-chain of secretory IgA)

Let's follow the process of synthesis and assembly of an immunoglobulin molecule in a plasma cell, referring to the diagram (Fig. 9-1) below.

FEATURES OF IMMUNOGLOBULIN SYNTHESIS

1) *Conventional mRNA molecules* are produced separately for the light and heavy chains, each containing uninterrupted coding sequences for V and C-regions. While the structure of the mRNA is conventional, some of the mechanisms involved in its synthesis (the DNA rearrangements discussed earlier) are quite unique to immunoglobulins. *Translation* of the message occurs on the ribosomes of the *rough endoplasmic reticulum (RER).*

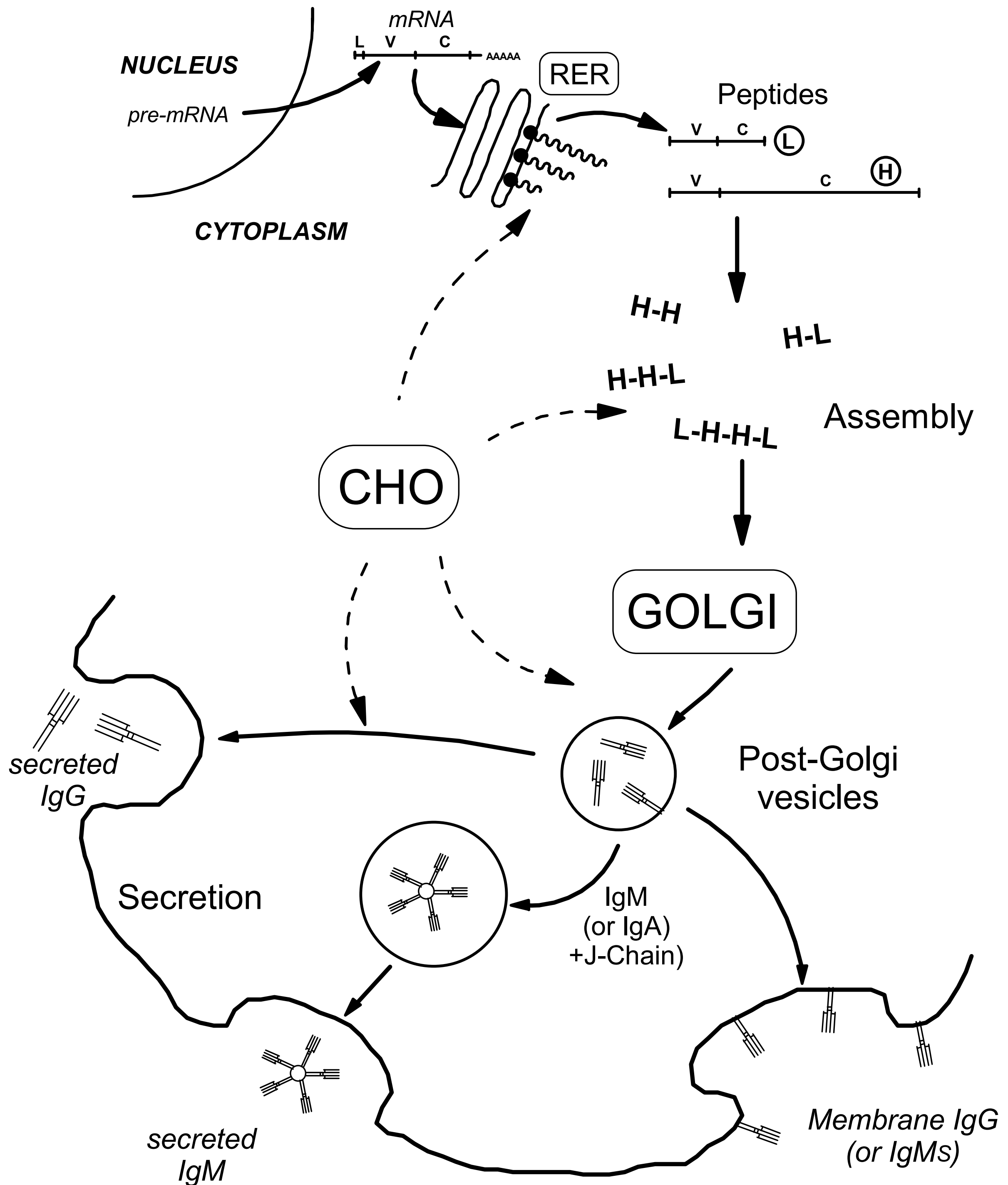


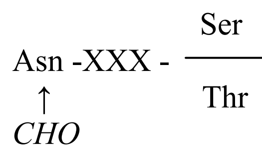
Figure 9-1

2) Nascent polypeptide chains are *translocated* across the membrane into the cisternae of the RER. As is the case for any secreted protein, this process requires the presence of the *leader polypeptide* or "signal sequence" at the amino terminus, which is enzymatically cleaved immediately after translocation.

3) *Assembly* of the heavy and light chains into a typical IgG-like subunit (H_2L_2) by formation of disulfide bonds is a *spontaneous* process - no specific enzymes are thought to be required, although a member of the HSP70 class of molecular "chaperonins" (the Heavy Chain Binding Protein, or BiP) is known to facilitate the proper folding of the heavy chains prior to their association with light chains. In normal plasma cells one sees balanced synthesis of H and L-chains, or a slight excess of L-chains (which are degraded intracellularly). Myeloma cells, on the other hand, often show markedly unbalanced synthesis with a large excess of L-chains secreted into the circulation which are then passed into the urine as Bence-Jones proteins. Some myelomas produce *only* light chains or *only* heavy chains; in the latter case (Heavy-Chain Disease) the H-chains are often defective, and typically have a deleted hinge region.

4) Assembled H_2L_2 molecules *move through the RER to the Golgi apparatus, and into the post-Golgi vesicles*. These vesicles then fuse with the external cell membrane and release their contents, resulting in *secretion of Ig from the plasma cell*.

5) Carbohydrate is added to H-chains in a well-defined order, beginning on nascent chains while they are still attached to ribosomes, and continuing throughout the process of movement through the cell right up to the point of secretion. Carbohydrate is added to asparagine residues which are part of a *specific recognition sequence*, namely an asparagine separated by one amino acid residue from a serine or threonine:



In normal antibody molecules carbohydrate is attached *only to H-chain*, and *only in the constant region*, because this recognition sequence is normally present only in C_H. Carbohydrate is a universal part of Ig heavy chains, and is thought to stabilize the three-dimensional structure of the Fc. [Note: In some myeloma proteins and other monoclonal immunoglobulins one sometimes finds V_H or V_L-associated carbohydrate in those cases where, through mutation, a recognition sequence happens to be present in the V-region. Carbohydrate will be attached to *any* accessible recognition sequence by the responsible enzyme system.]

6) Just prior to release of the immunoglobulin from the cell, two more events occur:

   a) The addition of a large final lump of carbohydrate. (The function of H-chain associated carbohydrate is still not fully understood, although it has been shown that its absence may affect the binding of Ig to Fc-receptors.)

b) In the case of IgM and polymeric IgA, the addition of the *J-chain*. The J-chain is appears to be required for the process of polymerization, although it can be experimentally removed from polymeric Ig without disrupting its structure.

NOTE: Not all Ig produced by a cell is necessarily secreted - some may be retained in the cell membrane and function as the B-cell's antigen receptor. The structural differences between secreted and membrane-bound forms of Ig are discussed below.

SECRETION OF IgA INTO EXOCRINE FLUIDS

We have accounted for all of the structural components of immunoglobulins except for the S-piece (*secretory piece*) associated with secretory IgA. The S-piece is *not* synthesized by the plasma cell which produced the immunoglobulin, but is added during the process of secretion into exocrine fluids. Let's look at the example of secretion of an IgA molecule into the gut in Figure 9-2 (although secretory IgA also appears in other exocrine secretions such as saliva and bile).

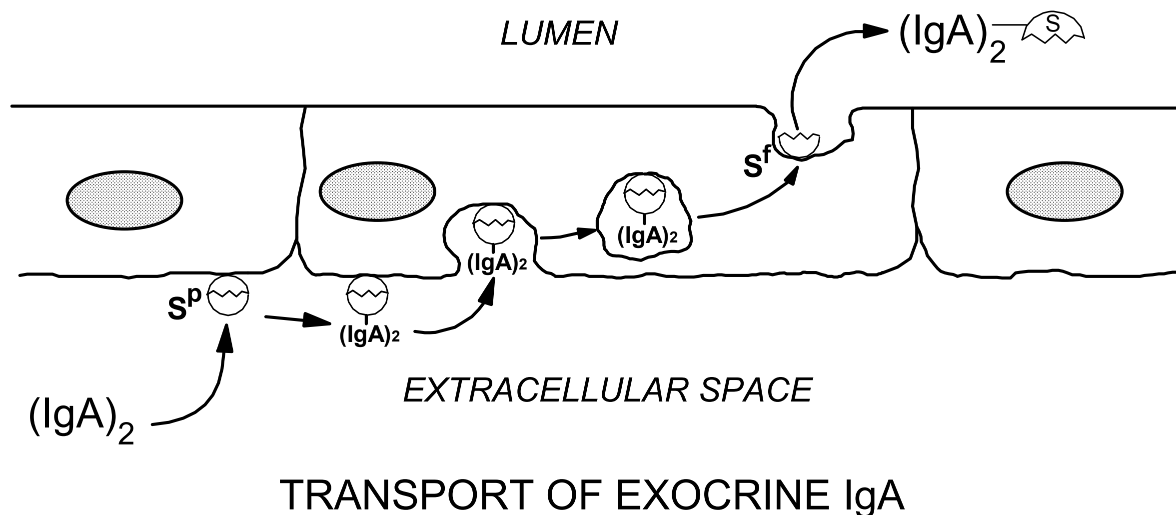


Figure 9-2

1) Polymeric IgA [mostly $(IgA)_2$, with a small amount of $(IgA)_3$], is secreted by plasma cells into the extracellular fluids of the lamina propria of the gut.

2) Epithelial cells lining the gut have *S-piece precursor* (labeled S^p in the figure above) in their abluminal membranes. This S^p behaves as a membrane receptor and *binds polymeric IgA*.

3) Polymeric IgA thus bound is internalized, transported to the luminal side of the cell, and is released into the lumen of the gut following proteolytic cleavage of the S^p into two fragments.

4) *Secretory IgA* now has conventional S-piece (one portion of the S^p) covalently linked to its H-chain; the rest of the S^p molecule remains in the epithelial cell membrane (as Sf, or S-fragment) where it is internalized and degraded.

5) *Only polymeric* IgA (and to a slight extent IgM) is transported in this manner, presumably because other Ig molecules simply do not bind to the S^p receptor on the epithelial cell. Monomeric IgA, the major serum form of IgA, is *not* secreted.

6) The presence of IgA in exocrine secretions can be of considerable importance in protection against infectious organisms. The normal route of entry of polio virus, for example, is the intestinal tract, and secretory IgA in this location confers a high degree of protection.

We've now seen two processes involving immunoglobulins which are both referred to as "secretion", and it is important not to confuse them.

- *Secretion of Ig from plasma cells*: Mature immunoglobulin molecules are "secreted" from the plasma cell into the extracellular space by the conventional process of exocytosis of post-Golgi vesicles. This same process is responsible for the release from cells of all "secreted" proteins.

- *Exocrine secretion of IgA*: Secretion of polymeric IgA into the mucus of the gut and other exocrine fluids is a distinct process of "secretion". This process, as we have seen, depends on the participation of the "secretory piece", or "S-piece", which is added to IgA by secretory epithelial cells. This process is unique to immunoglobulins, and IgA in particular.

KEY FEATURES OF ANTIBODY PRODUCTION

1) **One antibody-forming cell (AFC) cell produces only one kind of antibody**.

a) only *one* light chain isotype (κ *or* λ1 *or* λ2, etc).

b) only *one* heavy chain isotype.

c) only *one* kind of V_H and one kind of V_L; therefore, only *a single idiotype*.

One consequence of these features is that *antibody molecules are always symmetrical* with respect to their light and heavy chains.

(NOTE: There are some exceptions to the strict *one-cell-one-antibody* rule. One is the transient simultaneous synthesis by virgin B-cells of both IgM and IgD - the mechanism of this dual synthesis has been discussed in Chapter 8. Another situation which hardly qualifies as an "exception" is discussed under point [4], below. *Neither exception violates the basic principle of the symmetry of Ig molecules, or rules [a] and [c] above.)*

2) **Allelic exclusion**. While diploid cells have two copies of every immunoglobulin gene, only *one* of the two is expressed in a given B-cell or plasma cell for each of the light and heavy chain (the other allele is *either rearranged aberrantly* and cannot be expressed, *or is not rearranged* at all). This is an unusual and important feature of immunoglobulins (and the TCR); although recent work has shown that there exist a number of other genes that also exhibit this behavior.

   One of the consequences of allelic exclusion is that it ensures the *symmetry* of antibody molecules. Expression of *both* copies of the kappa locus (for example) would result in production of Ig moleculeswith *two* different Vκ regions, and therefore two different combining sites (remembering that the assembly of light and heavy chains is a random process). Such asymmetric molecules would have impaired biological activity (see the earlier discussion of affinity and avidity in Chapter 3), and would substantially dilute the pool of active bivalent molecules.

3) **Heavy chain class switching**. A particular antibody-forming cell can switch from production of IgM to IgG, from IgG to IgA, etc. The light chain does not change, nor does the V_H; *only the C_H changes*, so that the *original combining site* (and therefore idiotype) is now associated with a molecule of a *different class* or subclass.

   Switching is unidirectional--an IgG-producing cell *cannot* go back to production of IgM, for instance. The molecular basis of this irreversibility was discussed in the Chapter 8.

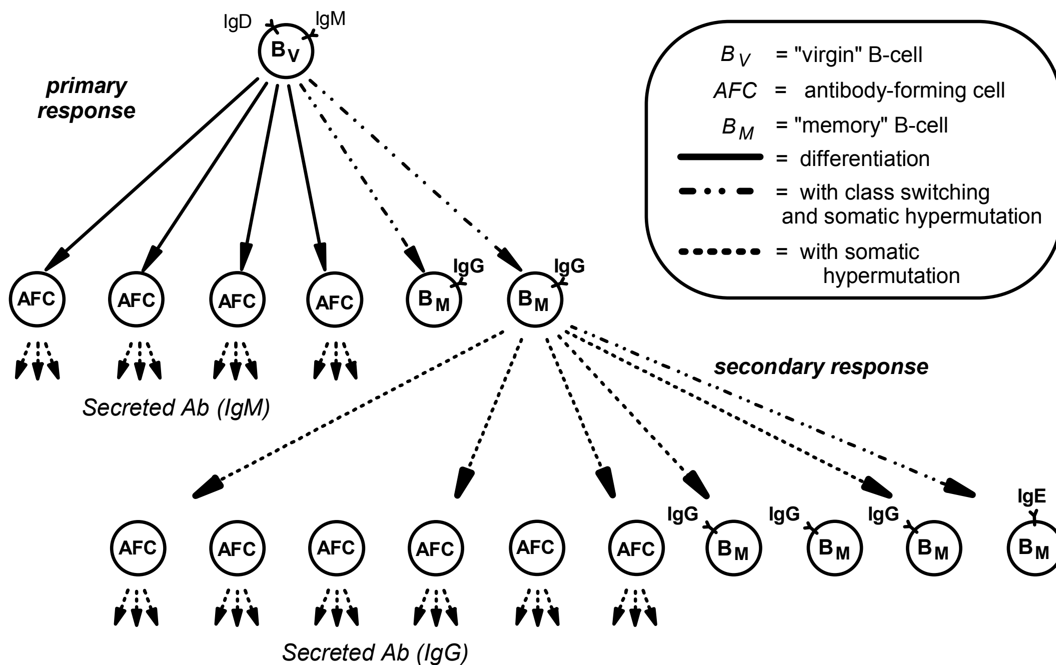
   Very rare myelomas have been found which produce two or more classes of immunoglobulin ("*double producers*"). These proteins generally differ *only* in their C_H and are considered to represent the results of class switching in these cells, the myeloma cells having gotten "stuck" during this process.

4) **Membrane-bound versus secreted immunoglobulins**. A virgin B-cell bears IgM (and possibly IgD) in its *membrane*; following stimulation it begins to *secrete* IgM into its surrounding environment. *These two forms of IgM are structurally different*. The mu heavy chain of membrane-bound IgM has an amino acid sequence at its carboxy-terminal end which anchors the molecule into the membrane; the secreted form of IgM has a *different* C-terminal sequence which lacks a membrane anchoring region. The membrane-bound form of IgM is also incapable of associating with J-chain and forming its normal pentameric structure; *membrane IgM, therefore, exists exclusively in monomeric* form (H_2L_2), also known as IgM_S or "sub-unit" IgM.

   The two forms of *mu* chain are synthesized via an alternative splicing scheme analogous to that which allows simultaneous IgM and IgD synthesis (as was discussed in Chapter 8). *Similar differences exist for secreted versus membrane-bound forms of IgG, IgA and IgE heavy chains.*

   A virgin B-cell produces *only* the membrane-bound form of IgM. As it develops into an antibody-secreting plasma cell it may transiently produce *both* membrane and secreted IgM, but it eventually produces only the secreted form. Likewise, a *memory B-cell* which synthesizes only membrane-bound IgG will shift to producing exclusively the secreted form of IgG as it develops into a plasma cell following secondary antigen stimulation.

Figure 9-3 shows the relationships between "virgin" B-cells (B_V, IgM-bearing), "memory" B-cells (B_M, shown as IgG-bearing, but can also be IgA or IgE-bearing), and antibody-forming cells (*AFC*, typically with little or no membrane-bound Ig). This illustrates some of the features of primary and secondary immune responses we have already discussed in Chapter 7 (*e.g.*, more rapid and efficient generation of AFC's from memory cells than from virgin B-cells), but also adds those membrane phenotypes of the participating cells which are a consequence of the molecular processes we have just covered (class switching from IgM to IgG, A or E). Note that the membrane-bound IgM (and IgD) which characterizes virgin B-cells is lost during the generation of *either* AFCs or memory cells.



IMMUNOGLOBULIN EXPRESSION BY B-CELLS AND THEIR PROGENY

Figure 9-3

5) **Somatic Hypermutation**. The V-regions of membrane Ig on memory cells typically express sequences *different* from those of the naïve B-cell from which they were derived, resulting in a marked increase in overall diversity which is reflected in the serum antibody response. This is the result of the accumulation of point mutations through a process called *somatic hypermutation*. As indicated by the broken lines in the figure above, this process occurs only during the generation of B-memory cells following isotype switching, and therefore affects only IgG, IgA and IgE.

   The mechanism involves the inactivation of the normal process of DNA editing, which exists to maintain sequence integrity during DNA replication. This inactivation, however, takes place only over a limited region *within and near the V-regions of both heavy and light chains*, excluding the constant regions. The point mutations which occur are, of course, random, but the only ones we will observe are the minority which happen to increase antibody affinity, thereby allowing the variant B-cells to be selected by antigen for continued proliferation and differentiation.

WHY CLASS SWITCHING?

B-cells start out with IgM and IgD on their surface; after antigenic triggering they lose their IgD and become IgM secretors. Most of them will subsequently switch from IgM to production of some other class of immunoglobulin during the course of an immune response. Why, then, do immune responses not start immediately with the final mixture of immunoglobulin isotypes? Why start every clone with IgM and go through the complex process of class switching?

There is clearly an advantage to ending up with a variety of isotypes represented in serum, because each will have its distinct set of biological activities. But the question still remains, why start everything off with IgM and only switch afterwards?

We can better understand this by recalling the phenomenon of **affinity maturation**:

a) As a consequence of clonal selection, the affinity of antibodies produced early in a response will always be lower than those produced later on (remember that the generation of the antibody repertoire is *random*).

b) Low affinity antibodies will tend to be less biologically effective than higher affinity ones because they bind less well to their target antigens.

c) The immune response can compensate for the *low affinity* of early antibodies by making *polymeric IgM* which will have a very *high avidity*, even with very low affinity combining sites.

d) However, IgM is very expensive for a cell to produce, it requires six times more raw materials and energy per molecule than IgG. Once the immune response has had time to become established using IgM antibodies, the process of affinity maturation can allow the expensive IgM to be replaced with more economical IgG-like antibodies, also gaining the additional benefit of a wide diversity of biological effector functions.

CHAPTER 9, STUDY QUESTIONS:

1. List each of the structural components which make up the various isotypes of human immunoglobulins. Describe the processes by which each is incorporated into a mature immunoglobulin.
2. What are the two distinct processes which are referred to as immunoglobulin "secretion"?
3. Why is allelic exclusion such a critically important feature of AFC's?

CHAPTER 10
BLOOD GROUPS: ABO AND Rh

The success of human blood transfusions requires compatibility for the two major blood group antigen systems, namely ABO and Rh. The ABO system is defined by two red blood cell antigens, A and B, whose presence or absence is determined by three alleles (A, B, O) segregating at a single genetic locus. An unusual feature of this system is the presence of serum IgM antibodies in healthy adults to whichever antigen (A or B) is *absent* from that individual's cells. The presence or absence of Rh antigens on red blood cells is determined by two alleles at another locus, Rh. Rh INCOMPATIBILITY between mother and infant may result in ERYTHROBLASTOSIS FETALIS, which can be prevented by passive immunization of the mother with anti-Rh antibodies (RHOGAM).

Many lives are saved throughout the world each year through the use of blood transfusions, by preventing death from loss of blood due to trauma, and by allowing performance of surgical procedures which would otherwise be impossible. However, it was not until the early nineteen hundreds that routine blood transfusion between humans became possible, following the discovery that genetically determined differences exist between the blood of different individuals. These differences must be identified and compatibility ensured before transfusions can be safely carried out.

Many genetic systems controlling blood groups in humans are known, and they have been of considerable importance in our understanding of human genetics, as well as in both clinical and forensic medicine. We will discuss briefly the major features of the two most important of these systems, those controlling the **ABO blood groups** and the **Rh factor**.

ABO BLOOD GROUPS

A and B antigens

The ABO blood groups are defined by the presence of two alternative antigens on red blood cells, determined by three alternative alleles at a single genetic locus. Two basic rules governing this system are as follows:

1) The blood "type" is defined by the presence of **two red blood cell antigens, "A" and "B."** RBCs of type A have the A antigen on their surface, those of type B have antigen B, type AB red cells bear *both* antigens, while type O cells bear *neither* antigen.

2) "Natural" antibodies called **isoagglutinins** exist in an individual's serum, directed against whichever of the A and B antigens is *not* present on that person's red cells (we will examine the source of these antibodies later).

The presence of ABO antigens and antibodies (*isoagglutinins)* in the four blood types is summarized below:

BLOOD TYPE	RBC ANTIGENS	SERUM ANTIBODIES	FREQUENCY
A	A	anti-B	40%
B	B	anti-A	10%
AB	A *and* B	none	5%
O	none	anti-A *and* anti-B	45%

The success of blood transfusions depends on ensuring the compatibility of the blood types between donor and recipient. If the *recipient* has antibodies to the infused red cells, these red cells will be rapidly destroyed, resulting in a potentially lethal **transfusion reaction**. Type A blood given to a type B recipient, for instance, can result in such a reaction, since the recipient's serum contains anti-A antibodies.

Blood typing & crossmatching

The presence of these antigens and antibodies can be readily detected by the **agglutination** reaction; mixing type A plasma (which contains anti-B antibodies) with type B red cells, for instance, results in agglutination of the red cells which can be easily observed. The blood type of any given individual can be determined in this manner, carrying out the agglutination reaction with a set of standard antibody-containing sera. Before a blood transfusion is given, in addition to choosing donor blood only of the same ABO type, direct **crossmatching** of donor and recipient is also generally carried out. This involves mixing donor RBCs with the recipient's serum to guarantee that the original typing of donor and recipient was correct, and to detect any possible unexpected agglutination reactions. (This is important because the complexity of the ABO system is considerably greater than we are discussing here, and many other relevant blood group systems exist as well.)

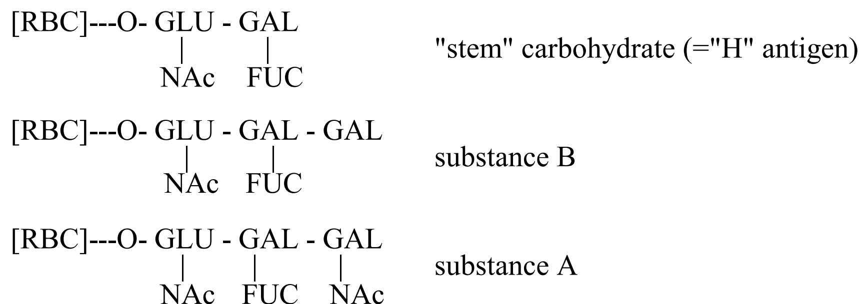
The primary cause of ABO mismatched transfusion reactions results from *destruction of donor red cells by the recipient's antibodies*; the reaction between donor antibodies and recipient cells is of less importance, since the small amount of antibody contained in the transfusion is generally diluted to harmless levels in the recipient. As a result, a type O individual (whose red cells bear neither antigen) is classically referred to as a **universal donor**, since his blood can fairly safely be given to a recipient with any ABO type; conversely, a person of blood type AB has been called a **universal recipient**, since his blood will contain no AB antibodies to damage any transfused red cells. Modern terminology more accurately refers to type O as a **universal red cell donor**, and to type AB as a **universal plasma donor** In general, however, every effort is made to assure complete compatibility

between donor and recipient ABO blood groups (as well as the Rh type which we will discuss below) provided that an appropriate donor is available.

The safety of blood transfusions which are not perfectly matched can be enhanced by transfusing packed red blood cells only, without the antibody-containing plasma; type O red blood cells are safe when given to any recipient, and the potential damage done by the donor's anti-A and anti-B antibodies may be avoided in this manner. But two relevant points should be noted. First, transfusions of whole human blood are actually now fairly uncommon, and the *vast majority of transfusions involve the use of separated blood components* such as packed RBC's, plasma, platelets, leukocytes or purified plasma proteins. Second, perfect matching of donor and recipient for ABO and Rh, even for transfusion of packed RBCs, is still the norm, except in unusual or emergency situations.

Structure of ABO antigens; "natural antibodies?"

Why are there so-called "natural" antibodies to A and B blood group antigens? A description of the nature and distribution of these antigens will help answer this question. The blood group substances A and B represent two modified forms of a "stem" carbohydrate present on red blood cells and other tissues. Their structures are shown below (where GLU is glucosamine, GAL is galactose or galactosamine, FUC is fucose, and NAc represents an N-acetyl group):



These same carbohydrates are also a common component of many foods we eat and many microorganisms in our intestinal tract. The immune system is therefore constantly exposed to these antigens, and responds by making an effective humoral response. Since the immune system does not in general respond to antigens which are a normal part of "self" (see Chapter 18, TOLERANCE), a type B individual does not make antibodies to the B blood group substance, although the response to the type A antigens is robust. The net result is the production of antibodies, mostly of the **IgM** class, to whichever of these substances is *not* present on an individual's red blood cells. (It should be noted that such complex carbohydrates are a typical example of *thymus-independent antigens*, which generally elicit only IgM antibodies, discussed in Chapters 13 and 15.)

It is important to remember that the A and B blood group substances are present not only on red blood cells, but also in *virtually every other tissue*. They are therefore **important transplantation antigens** and must be taken into account together with HLA tissue-typing (see Chapter 11) when organ transplantation is performed. This is particularly important

given the fact that substantial levels of anti-A and anti-B antibodies (isoagglutinins) may be normally present, depending on the recipient's blood type.

Genetics of ABO

The presence of A and B carbohydrates in our tissues is determined by three alleles at a single genetic locus. One allele encodes an enzyme which produces the A substance, another the B substance; and when both of these alleles are present in a heterozygote both carbohydrates are made. The third allele, O, behaves essentially as a "null" allele, producing neither A nor B substance. Thus, while the ABO system yields only *four blood types (phenotypes),* there are six possible *genotypes*:

Genotype	*Blood Type (Phenotype)*
A/A	A
A/O	A
B/B	B
B/O	B
A/B	AB
O/O	O

Only a single genotype can produce the phenotype AB, namely the heterozygous state A/B. Likewise, type O individuals must be homozygous O/O. However, type A or type B individuals can be either homozygous or heterozygous, the O allele being effectively recessive since it does not contribute either of the two antigens.

The inheritance of the ABO blood groups follows simple Mendelian rules. For instance, a homozygous type A mother and a type AB father (below, left) can yield only two kinds of offspring, type A (genotype A/A) or type AB (genotype A/B). A heterozygous type A and a heterozygous type B, on the other hand (below, right), can yield four genotypes and four corresponding phenotypes.

A/A X A/B		A/O X B/O				
						parents
A/A	A/B	A/B	A/O	B/O	O/O	*offspring*
0.50	0.50	0.25	0.25	0.25	0.25	*frequency*

Rh BLOOD GROUPS

Genetics

While many blood group systems are known other than the ABO system, the Rh system is of special importance,. This was originally defined by a rabbit antibody directed against the red blood cells of Rhesus monkeys, an antibody which turned out to be capable of distinguishing between the red blood cells of different human individuals. In simple terms, this system is defined by the presence or absence of a single red blood cell antigen, representing the two blood types Rh+ and Rh-. These are determined by two alleles at a single locus, which segregate independently of the ABO blood group locus. Thus an Rh+ individual may be homozygous (+/+) or heterozygous (+/-), while an Rh- individual must be homozygous (-/-).

The Rh^- blood type is relatively uncommon, representing less than 15% of the population. Since Rh segregates independently of ABO, one can readily calculate the frequency of any given combination of ABO type and Rh type. If type A represents 40% of the population, and Rh- only 15%, then the frequency of type A, Rh^- individuals is given by:

$$0.40 \times 0.15 = 0.06$$

or ~6% of the population.

So-called "natural" antibodies to Rh do not exist in humans, as they do for the AB antigens. However, Rh+ cells infused into an Rh negative recipient can give rise to a strong antibody response, mainly of the IgG class, which can result in dangerous reactions to subsequent transfusions. Blood typing and cross-matching are therefore important to ensure compatibility for the Rh factor as well as ABO. However, unlike the A and B antigens, the *Rh antigens are present only on red blood cells*. Therefore, while they are important for blood transfusion, *they do not normally play a role in organ transplantation*, and Rh typing of organ donors and recipients therefore not a significant consideration.

Rh-incompatibility; RhoGAM Therapy

The Rh factor assumes a special importance in maternal-fetal interactions. A mother who is Rh^- can bear an Rh+ child if the father is Rh+ (either homozygous or heterozygous). Since there are no natural anti-Rh antibodies, this generally poses no special risk for the first pregnancy. At the time of birth, however, tissue damage resulting from the separation of the placenta from the uterine wall can result in a significant amount of fetal blood entering the maternal circulation; which may stimulate a *strong IgG anti-Rh response* in the mother.

If the same mother then bears a *second* Rh+ child, the existing anti-Rh antibodies can cross the placenta during the pregnancy and destroy fetal red blood cells. The ensuing damage to various organs results in the potentially dangerous condition **Erythroblastosis Fetalis** (also known as *Hemolytic Disease of the Newborn*, or **HDN**). This can be diagnosed prenatally by carrying out amniocentesis, and examining the amniotic fluid for the presence of free hemoglobin and its degradation products. Various approaches can be used during and after birth to rescue the infant, including **exchange transfusion**, complete replacement of the infant's blood to remove the anti-Rh antibodies and provide undamaged red blood cells.

However, the production of anti-Rh antibodies in an Rh- mother can often be prevented by administering anti-Rh immune globulin (*e.g*. **RhoGAM)** into the mother, typically at around 28 weeks of gestation and again within 72 hours of the birth of her Rh+ baby. By mechanisms which are still not fully understood, these antibodies greatly reduce the likelihood of sensitization of the mother's immune system by the Rh+ erythrocytes. If this procedure, developed in the 1960's, is successfully carried out *during each Rh+ pregnancy*, anti-Rh antibodies are not produced by the mother, and subsequent pregnancies will not be at risk.

While Rh incompatibility is of considerable clinical significance, it should be noted that *not all untreated incompatible pregnancies result in disease*. Only a small fraction of

incompatible pregnancies actually result in the production of maternal anti-Rh antibodies, and in only a fraction of these cases is there significant damage to the newborn.

What about ABO incompatibility?

If maternal antibodies to red blood cells can damage the developing fetus, why is incompatibility for ABO blood groups not as dangerous as Rh-incompatibility, particularly since ABO isoagglutinins normally exist in mothers which could potentially damage the infant even during a first pregnancy? The answer lies in the isotype of antibody produced in the two cases. Anti-Rh-antibodies are mainly IgG (typical of anti-peptide responses) which is capable of crossing the placenta and entering the fetal circulation. The natural antibodies (isoagglutinins) to A and B blood group substances, however, are mostly of the IgM class (typical of anti-carbohydrate responses) and therefore do not cross the placenta.

IgG antibodies to the A and B blood group antigens may develop in some individuals, and the resulting ABO incompatibility actually accounts for about two thirds of all *discernable* cases of HDN; such cases, however, are generally very mild and require little or no treatment. Thus, while ABO incompatibility is actually much more common than Rh incompatibility, it is much less likely to cause significant d
isease. In fact, *it appears that the presence of ABO incompatibility between mother and fetus may confer some protection against the development of clinical Rh incompatibility*.

CHAPTER 10, STUDY QUESTIONS:

1. How many potential genetic crosses exist in which parents with *identical* ABO phenotypes can produce children who *differ* from one another in their phenotypes?
2. Is the Rh antigen a T-DEPENDENT or T-INDEPENDENT ANTIGEN? (See Chapter 15).
3. Maternal-fetal ABO incompatibility, which is much more common than Rh incompatibility, may *protect* against the development of anti-Rh antibodies in Rh-incompatible pregnancies. Suggest an explanation for this phenomenon.

CHAPTER 11
CELL-MEDIATED IMMUNITY AND MHC

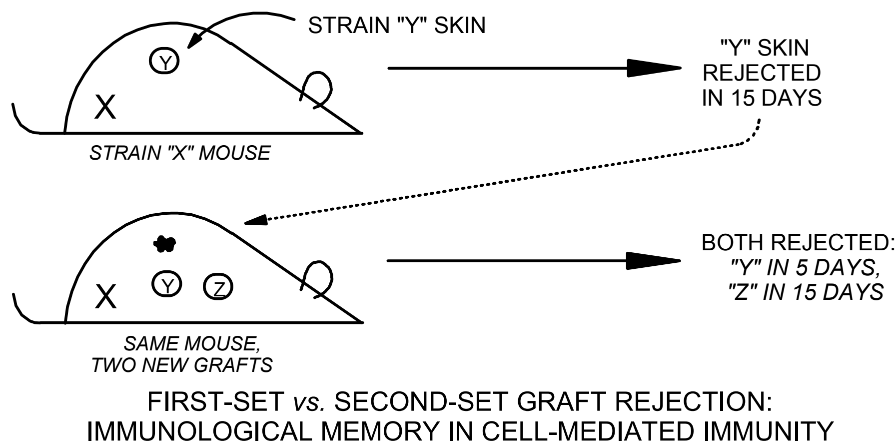
See APPENDIX (10) INBREEDING; (11) MIXED LYMPHOCYTE REACTION (MLR)

Graft rejection is a manifestation of CELL-MEDIATED IMMUNITY (CMI), as can be demonstrated by adoptive transfer experiments; antibodies generally contribute little to the rejection of grafted tissues. Simple rules predict the acceptance or rejection of *alloografts* between inbred mice. Although these specific rules are not valid in humans (or other outbred populations), a common fundamental principle holds, namely, that a graft will be rejected if the host's immune system recognizes a *foreign* antigen on the graft. The most important antigens contributing to graft rejection in vertebrates are those encoded by the MAJOR HISTOCOMPATIBILITY COMPLEX (MHC), a cluster of genes known as H-2 in mice and HLA in humans; within this complex are encoded several CLASS I and CLASS II molecules, with distinct structures and biological functions. The basic rules of immune recognition and reactivity are illustrated by an analysis of GRAFT-VERSUS HOST (GvH) reactions.

TRANSPLANT REJECTION: CELL-MEDIATED IMMUNITY

The greatest barrier to our ability to transplant tissues and organs between individuals is rejection by the immune system: grafts between genetically different individuals are generally rejected. Using skin grafts between mice as a model system, let's examine the basic immunological and genetic features of such a rejection reaction.

If we graft a patch of skin from one mouse of strain Y onto another mouse of strain X (an *allograft*), the graft will heal and may remain viable for a week or more, but at the end of about 15 days it will have been rejected -- only a bit of scar tissue will remain at the site (as indicated in Figure 11-1 below). This is known as **first set** rejection (*i.e.* primary immune response). A simultaneous graft from another strain X mouse (an *isograft* or *syngeneic* graft, not shown in the figure) will heal and remain viable and intact indefinitely.



FIRST-SET *vs.* SECOND-SET GRAFT REJECTION:
IMMUNOLOGICAL MEMORY IN CELL-MEDIATED IMMUNITY

Figure 11-1

We then take the same mouse which has rejected the first graft, and place on it two more grafts, one from strain Y and another from a third, unrelated mouse strain Z. The Z graft is rejected as was the original Y graft, in about 15 days. The new Y graft, however, is rejected in five days, much more rapidly than the first time. This is known as **second set** rejection (*i.e.* secondary immune response).

Graft rejection is an immunological phenomenon, defined by the following properties.

i) It shows **specificity**. Graft Y (an *allograft*) is rejected while the *isograft* X is not effected.

ii) It displays **immunological memory**. Graft Y is rejected in "second set" fashion the second time around, while the simultaneous grafting of skin from strain Z is rejected in "first set" fashion (this is also a reflection of immunological *specificity*).

iii) It is **systemic**. While rejection occurs in a particular location (at the site of the grafted skin in this example), the *ability* to reject a graft is not localized, either in first set or second set tempo. The first or second graft of Y skin could have been placed *anywhere* on the recipient (apart from certain immunologically "privileged" sites; see Chapter 18), and the result would have been the same. The systemic nature of immunological responsiveness is a consequence of the continuous and rapid movement of cells of the immune system throughout the body, which we will discuss more fully in Chapter 16.

GRAFT REJECTION: MANIFESTATION OF CELL-MEDIATED IMMUNITY

The above experiment showed us that graft rejection is an immunological process, and we can now ask whether it is a manifestation of HUMORAL or CELL-MEDIATED immunity (*i.e.* is it mediated by antibodies or not). To answer this question we carry out an ADOPTIVE TRANSFER experiment, transferring either serum or spleen cells from a strain X mouse which has already rejected a Y skin graft, into two untreated strain X recipients. We then graft each of the two recipients with strain Y skin, and observe how long it takes for the grafts to be rejected. These are "first set" grafts, and we would normally expect them to be rejected in about 15 days.

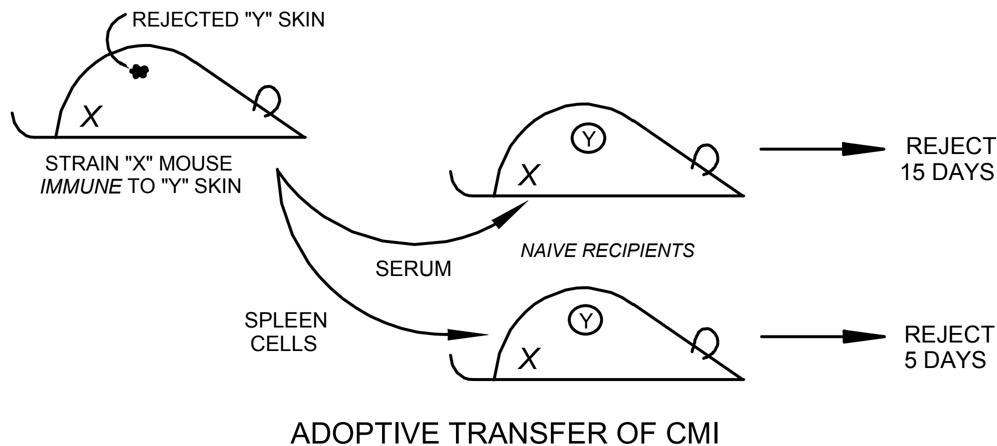


Figure 11-2

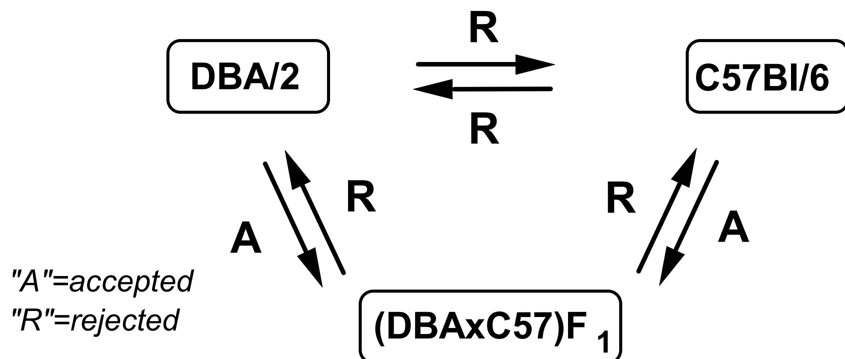
We find, however, that the mouse treated with immune spleen cells rejects the graft in "second set" fashion (in about *five* days) while the one treated with immune serum rejects it in the expected "first set" fashion (*fifteen* days). Thus, we find that *immunity to the skin graft has been transferred by spleen cells but not by serum*. This defines graft rejection as a CELLULAR immune response, or a manifestation of *cell-mediated immunity* (CMI).

(If we had been studying a different kind of immune response, namely protective immunity to Pneumococcus in mice, we would have gotten a different result [See Fig. 2-1]. In that case immunity would have been transferred by *either* immune spleen *cells o*r immune *serum*, which would define it as HUMORAL immunity. Humoral and cellular immunity are *both* transferable by spleen cells, but *only* humoral immunity is transferable by serum or other antibody-containing fluids.)

With some exceptions ("hyperimmune rejection" of kidney grafts, for instance) *circulating antibody contributes little to rejection of grafted tissues*. In some instances, in fact, the presence of antibody can *protect* a graft from rejection, a phenomenon known as **immunological enhancement** (and such antibodies are referred to as "enhancing" antibodies). The mechanism of this phenomenon is complex, and may involve "hiding" of histocompatibility antigens from the host's cellular response when they are bound with antibody.

GENETIC RULES OF TRANSPLANTATION

Let's examine (Figure 11-3) the pattern of rejection or acceptance of skin grafts between three inbred strains of mice, one of strain C57Bl/6, another of strain DBA/2, and the third an F_1 hybrid between these two inbred strains. The arrows indicate the direction of the graft; an "**A"** indicates the *acceptance* of a particular graft while an "**R"** indicates *rejection*. (NOTE: The rules shown in this figure hold for inbred mice, but *not* for outbred organisms, including humans.)



TISSUE GRAFT REJECTION IN INBRED MICE

Figure 11-3

The key features of immunological graft rejection are summarized below:

1) *A graft is rejected if a foreign ("non-self") antigen is recognized*. Grafts between two unrelated inbred strains, or between two individuals of a non-inbred species, will be rejected.

2) *Grafts from an F1 hybrid onto a parental inbred strain are rejected.* The antigens of *both* parental strains are expressed on the F1, and each will be seen as foreign by the other parental strain.

3) *Grafts from either inbred parent strain onto an F1 will be accepted.* No foreign antigen can be recognized in such a graft.

4) *The antigens triggering graft rejection are cell surface glycoproteins.* Most of these are present on *all* tissues of an organism, and are known as HISTOCOMPATIBILITY ANTIGENS.

5) *There are many genes which encode histocompatibility antigens (more than 30 named genes in mice).* Any gene encoding a *polymorphic* protein is potentially a histocompatibility locus. (NOTE: Only *polymorphic* loci can contribute to allograft rejection, since a *monomorphic* gene product will not be foreign to any individual of the species.)

6) *In any given vertebrate species, one single histocompatibility "gene" predominates in promoting graft rejection.* This "gene" is known generally as the MAJOR HISTOCOMPATIBILITY COMPLEX (MHC), and has different specific names in different species.

HUMAN	-	HLA (***H***uman ***L***eukocyte ***A***ntigen)
MOUSE	-	H-2 (***H***istocompatibility locus # ***2***)

The MHC is, in fact, not a single gene, but is actually a *closely linked complex of many genes*, dozens or more--hence its name. These genes are highly polymorphic in most species, accounting for the fact that *allografts in humans (and other non-inbred species) are almost always rejected*.

The many histocompatibility genes *outside* of the MHC are collectively known as *minor histocompatibility loci*. Differences at the MHC will always cause rapid graft rejection, regardless of the status of minor loci. Differences at minor loci, however, even *many* of them simultaneously, will *not* cause as rapid rejection as a difference in the MHC itself.

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) OF MICE AND HUMANS

A simplified diagram of the **H-2 complex** of mice compared with the **HLA complex** of humans is shown below:

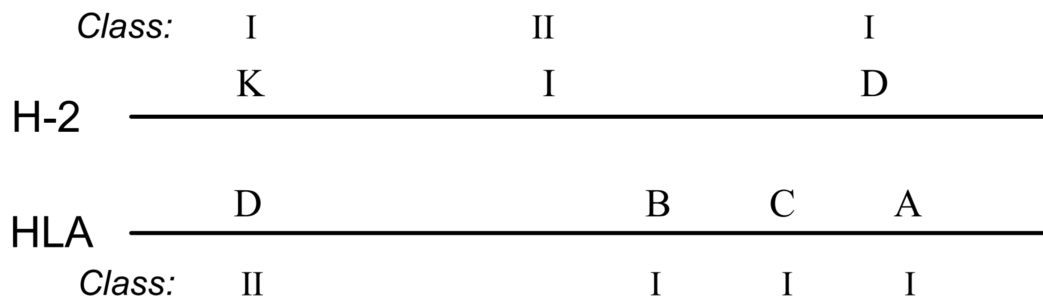


Figure 11-4

Three regions can be distinguished in the H-2 complex, *each of which is itself a complex of multiple gene loci:*

The K and D regions encode Class I molecules. Class I molecules of the MHC are *expressed on all mouse cells* and the major targets for recognition and rejection of foreign grafts. Skin grafts between strains of mice differing only in the I-region (Class II) will be rejected only slowly, if at all, while differences in K or D will result in rapid rejection. In humans, Class II differences play a larger role in inducing rejection, which will be discussed later.

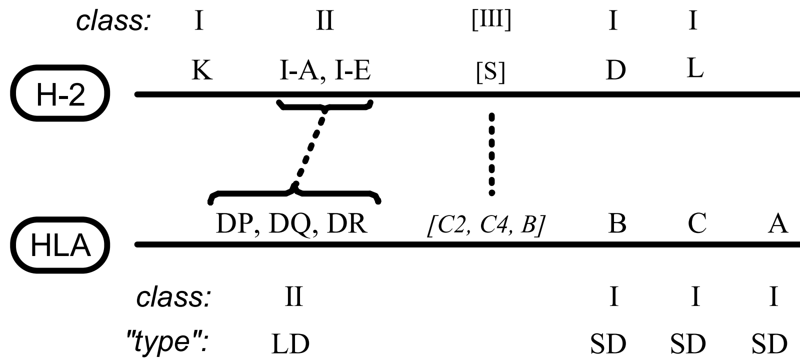
The I-region encodes Class II molecules. Class II molecules of the MHC are *expressed only on some cells,* and are required for the process of *antigen presentation* to "helper" T-cell (discussed in Chapters 12 and 15). In mice these antigens are referred to as Ia antigens (for "I-region Antigens"), a term sometimes loosley applied to human Class II molecules.

The I region of mice was shown many years ago to contain genes which control the ability to generate immune responses to particular antigens, genes were called **"Ir genes"** or *"Immune Response Genes;"* this is the feature of the I-region that originally gave it its name. It was subsequently shown that these *"Ir" genes are the Class II genes themselves*, and the mechanism by which they regulate immune responsiveness involves their participation in the process of antigen presentation (see Chapters 12 & 15).

The HLA complex of humans is organized slightly differently, but in a homologous fashion:

> **The A, B and C regions of HLA encode Class I molecules,** *which are expressed on all nucleated cells*. While Class I molecules are the major targets for graft rejection, Class II molecules can play a much more important role in human graft rejection than they do in mice. Tissue typing for Class I antigens in humans is traditionally carried out by using *antibodies* specific for the various allelic forms; these antigens are therefore referred to as *Serologically Determined, or SD antigens*.
>
> **The D-region of HLA encodes Class II molecules (***which are expressed only on some cells)***,** and includes several defined subregions, namely DP, DQ and DR (as seen below). These genes are capable of regulating certain kinds of immune responses (typically autoimmune conditions including "rheumatic" diseases), in a manner analogous to the I-region of mouse H-2. Antigens of the D-region of HLA are traditionally not detected by antibodies (although there are now exceptions), but are determined using the MIXED LYMPHOCYTE REACTION (MLR) (see APPENDIX 11). D-region antigens are therefore referred to as *Lymphocyte-Determined (LD) antigens*, to distinguish them from the *SD antigens* of the A, B and C regions.

Shown below in Fig. 11-5 is a more detailed diagram of H-2 and HLA, showing a few additional features of each, as well as some of the relationships between the two:



MAJOR HISTOCOMPATIBILITY COMPLEX OF MOUSE AND HUMAN

Figure 11-5

Several new features are evident:

The D-region of H-2 is seen as containing two sub-regions, D and L, both of which encode *Class I* antigens. The D-region of HLA, which encodes *Class II* antigens, can ce seen to contain *three* subregions, namely DP, DQ, and DR, mentioned above.

An additional region is shown in *both* H-2 and HLA which codes for the *C2, C4 and B components of complement* (this region is called the S-region in mouse). These genes are also referred to as Class III genes, although the reason for these complement component genes (and several other unrelated genes) being located in the middle of the MHC remains a puzzle.

While there are obviously differences in the organization of the MHC of mouse and man, their common features are the most important. They both contain genes determining Class I and Class II antigens; they are both highly polymorphic; they both encode glycoproteins which are the major targets for graft recognition and rejection (Class I) as well as mediators of cell cooperation in immune responses (Class II).

GRAFT-VERSUS-HOST REACTION (GvH)

The rules of transplantation as outlined above (see Fig. 11-3) tell us that a *skin graft* from a C57Bl/6 mouse onto a (C57Bl/6xDBA/2)F1 recipient will be accepted indefinitely, with no resulting harm to either graft or host. However, if we transfer *spleen cells* in this same genetic combination, we see something quite different--*the recipient will rapidly sicken and die*. This phenomenon is the result of an immune reaction carried out by the grafted cells against histocompatibility antigens present in the recipient, and is known as a **Graft-versus-Host (GvH**) reaction. The GvH reaction is of great importance in many human tissue and organ grafts, and we need to understand the principles underlying its production.

In order for a GvH reaction to occur after tissue or organ transplantation, the following *three conditions must hold*:

1) **The grafted tissue must contain immunocompetent cells**. Grafted skin cannot cause GvH simply because it does not contain cells capable of carrying out immune reactions. Spleen, however, does contain appreciable numbers of immunocompetent cells, as does lymph node, and both can therefore initiate a GvH reaction in an experimental situation. While neither spleen nor lymph node cells would normally be transferred between humans, transfer of other lymphocyte-containing tissues such as blood or bone marrow

may be capable of initiating GvH reactions. In addition, some organ grafts may contain sufficient numbers of lymphocytes to initiate GvH reactions, notably liver, intestine and lung.

2) **The graft must be capable of recognizing foreign antigens on host tissue**. Spleen cells from an F1 mouse injected into a parental strain recipient, for instance, will not give rise to a GvH, as the host tissues bear no antigen which is foreign to the F1 graft. In this example, in fact, the grafted spleen cells would be rapidly destroyed (since these cells *do* bear antigens foreign to the host's immune system). In the human situation, only a graft between identical twins would fail to be able to recognize any foreign antigens in the host.

3) **The recipient must be incapable of rejecting the grafted tissue**. In the mouse example cited above (C57Bl/6 spleen cells into an F1 recipient), the host cannot reject the transplanted spleen cells because no *foreign antigens are recognized* (this is the same reason it cannot reject a skin graft in that genetic combination). However, other reasons may also exist for failure to reject the graft. For example, injection of C57Bl/6 spleen cells into a *newborn* DBA/2 mouse will result in fatal GvH. In this instance the newborn mouse cannot reject the graft because its *immune system is not sufficiently developed* – such a graft would *not* result in a GvH if given to an *adult* DBA/2 mouse because it would be rapidly rejected. In humans, however, the immune system of a newborn is perfectly capable of rejecting a foreign graft (see Chapter 17). A third example is that where the host immune system is defective due to a *congenital immunodeficiency or immunosuppression*, in either an experimental or clinical setting. If an adult DBA/2 mouse is subjected to sublethal X-irradiation, or treatment with anti-lymphocyte serum (a powerful immunosuppressant), injecting C57Bl/6 spleen cells *will* result in a GvH reaction. Likewise, transfusion of whole blood into a human infant suffering from a congenital T-cell immunodeficiency may result in a fatal GvH [see Chapter 20]).

CLINICAL SIGNIFICANCE OF GvH

GvH reactions have been very useful to immunologists studying the genetics and mechanisms of the cellular immune response, but they have also been of considerable clinical significance. A classic example is that of **bone marrow/stem cell transplantation** which *has long been potentially dangerous due to the presence of immunocompetent cells that can carry out a GvH reaction*. Methods for removing such cells prior to transplantation and for controlling the resulting GvH have become much more sophisticated and effective in recent years, but stem-cell transplantation remains a fairly high-risk procedure.

Another example is that of **transfusions of whole blood**, which, of course, contain immunocompetent T-cells. Most commonly, such transfusions pose little danger of producing GvH disease, since the recipient's immune system will recognize and reject the foreign T-cells ("Rule 3", above, does not hold). However, if the recipient is immunodeficient, as in the example above, a fatal GvH reaction may ensue from what would otherwise be a harmless transfusion. In such cases the risk, if it is recognized, can be eliminated by X-irradiation of the transfused blood, which eliminates the lymphocytes without damaging red blood cells or other important blood components.

Whole blood transfusion may also pose a significant risk for GvH if they happen to be from an HLA-homozygous donor into a recipient heterozygous for the same haplotype as the

donor. This is ordinarily a rare occurrence in most populations which are highly polymorphic for HLA, but occurs more frequently in some populations with more restricted heterogeneity, particularly in the case of transfusions between first-degree relatives (parent/offspring, and siblings).

CHAPTER 11, STUDY QUESTIONS:

1. Describe the cellular basis for the difference between "*first set*" and "*second set*" GRAFT REJECTION?
2. Why do the simple RULES OF TRANSPLANTATION between inbred mice (Fig. 11-3) not apply to humans? What rules *do* apply to humans?
3. How many human chromosomes carry genes encoding HISTOCOMPATIBILITY ANTIGENS?
4. What are the differences between CLASS I and CLASS II MHC molecules?
5. Draw a diagram showing the rules for the ability of cell transfers between inbred strains to generate GvH REACTIONS (analogous to Figure 11-3).

CHAPTER 12
CELL INTERACTIONS IN CELL MEDIATED IMMUNITY

SEE APPENDIX (11) MIXED LYMPHOCYTE REACTION

The diverse manifestations of CELL-MEDIATED IMMUNITY (CMI) include several mechanisms by which target cells may be killed. The induction of one of these mechanisms, namely killing by CYTOTOXIC T-CELLS, involves the participation of two distinct, antigenically precommitted lymphocytes (T_H- and T_C -cellCELLS) as well as non-specific antigen-presenting cells (APCs, typified by DENDRITIC CELLS and MACROPHAGES). T_H cells can be subdivided into two major sub-types, T_{H1} cells which promote inflammatory reactions and CMI, and T_{H2} cells which largely provide "help" to B-cells in generating humoral immunity.

T-cell receptors recognize antigenic peptides only when they are associated with MHC molecules, which accounts for the phenomenon of MHC-RESTRICTED RECOGNITION. The existence of two DISTINCT CELLULAR PATHWAYS by which CLASS I and CLASS II molecules become associated with antigenic peptides is described. Resistance to the intracellular bacterium LISTERIA serves to illustrate a *distinct* mode of lymphoid cell interactions and cell-mediated effector functions which does not depend on T_C cells.

All those immune reactions which can *not* be transferred by serum or other antibody-containing fluids are referred to as CELL MEDIATED immunity, or CMI. CMI is initiated by various kinds of T-cells, and both T-cells and other cell types (notably macrophages) may act as effector cells in such reactions. Because of its characteristic slow time course, CMI is also referred to as DELAYED-TYPE HYPERSENSITIVITY or DTH (although this term is often used to refer specifically to one particular type of skin reaction described below). In Chapter 14 we will discuss some of the characteristic surface markers of known T-cell subsets. But remember that **all T-cells (like B-cells, but unlike macrophages or any other cells) are clonally precommitted, and have antigen-specific receptors in their membranes.** Remember also that **T and B-cells use different molecules for antigen-recognition**.

Examples of CMI Reactions	Effector T-cells
1) **Graft rejection**. The major barrier to successful tissue and *organ transplantation* is immune rejection. Antibodies are not the primary mediators of such rejection, although they can be involved in various ways (as we will see later).	$\mathbf{T_{H1}/T_C}$
2) **Resistance to certain infectious diseases**. While humoral immunity is important for protection against many bacterial infections, immunity to certain pathogens, notably *fungi* and intracellular parasites (including *viruses* and intracellular bacteria) depends primarily on the cellular immune system.	$\mathbf{T_{H1}/Tc}$
3) **DTH skin reaction**. The *tuberculin skin test* is often used as the classic example of delayed-type hypersensitivity reactions or cell-mediated immunity. It is one manifestation of the mechanism of graft rejection and immune resistance to intracellular parasites.	$\mathbf{T_{H1}}$

4) **Graft-versus-host reaction**. *GvH* has been discussed in Chapter 11, and is of both experimental and clinical interest. **T_{H1}/T_C**

5) **Contact dermatitis**. Sensitivity to poison oak, certain drugs, and other environmental substances (*e.g*., cosmetics) involve CMI skin reactions against a reactive hapten coupled to an endogenous carrier. While the carrier is normally regarded by the immune system as"self, the conjugate may highly immunogenic. One way to distinguish contact dermatitis from IgE-mediated allergy ("immediatehypersensitivity") is by comparing the *time course* of development of the reactions. **T_{H1}/T_C**

6) **Mixed lymphocyte Reaction (MLR).** This *in vitro* manifestation of cell-mediated immunity has been of considerable importance in MHC tissue-typing in preparation for transplantation, and is described in more detail in APPENDIX 11. **T_{H1}**

(NOTE: The more recently defined T_{H17} cell is discussed in Chap. 14.)

THREE MECHANISMS FOR CELL-MEDIATED TARGET CELL KILLING

1) ***T_C -mediated lysis (which is the principle topic of this chapter)***

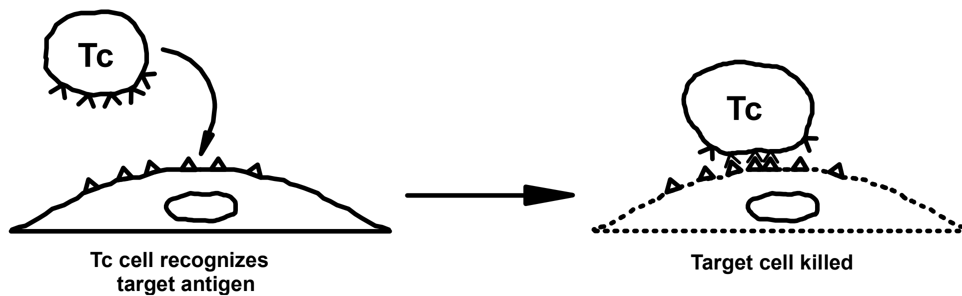


Figure 12-1

Activated T_C cells specific for a cell-surface antigen, bind to the target and cause lysis, as seen in Figure 12-1 (these cells are *Ag-specific, clonally committed* T-cells). This binding requires ASSOCIATED RECOGNITION of the foreign antigen together with self class I MHC antigens; this phenomenon is discussed in more detail later in this chapter.

2) ***NK-killing***

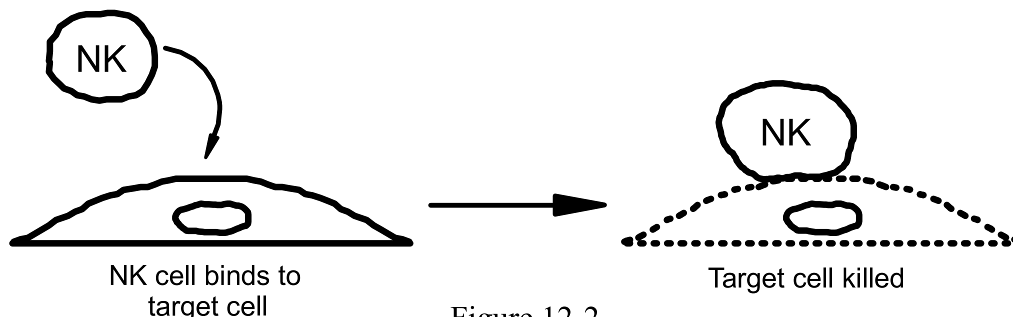


Figure 12-2

Another cell which can kill target cells is the NK or "Natural Killer" cell. NK cells were first detected using *in vitro* assays, through their ability to kill certain tumor cell targets (schematized in Figure 12-2). Unlike T_C cells, which utilize antigen-specific receptors to recognize I MHC Class I-associated peptides on target cells, NK cells kill targets based on their expression of *abnormally low levels of MHC-I molecules* (regardless of the particular peptides associated with them), or *abnormal carbohydrates*. Tumor cells which *under*-express MHC molecules, or virus-infected cells which may down-regulate MHC-I expression (thereby avoiding T_C-based killing) may thus become targets for NK killing.
NK cells are not induced by prior exposure to the tumor cells, but are constitutively present. They are members of a population of white blood cells known as **Large Granular Lymphocytes** (LGL), originally named "null" lymphocytes due to their lack of the membrane markers (notably Ig and TCR/CD3) which defined B-cells and T-cells.

3) Antibody Dependent Cell-mediated Cytoxicity (ADCC).

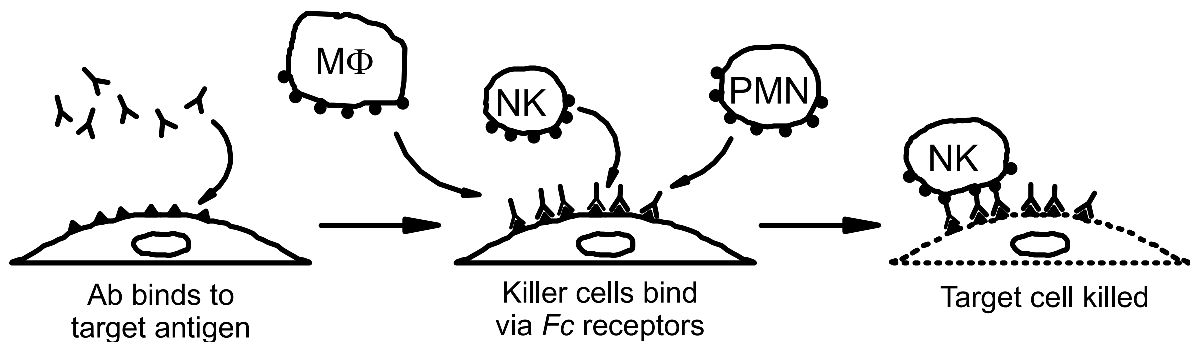


Figure 12-3

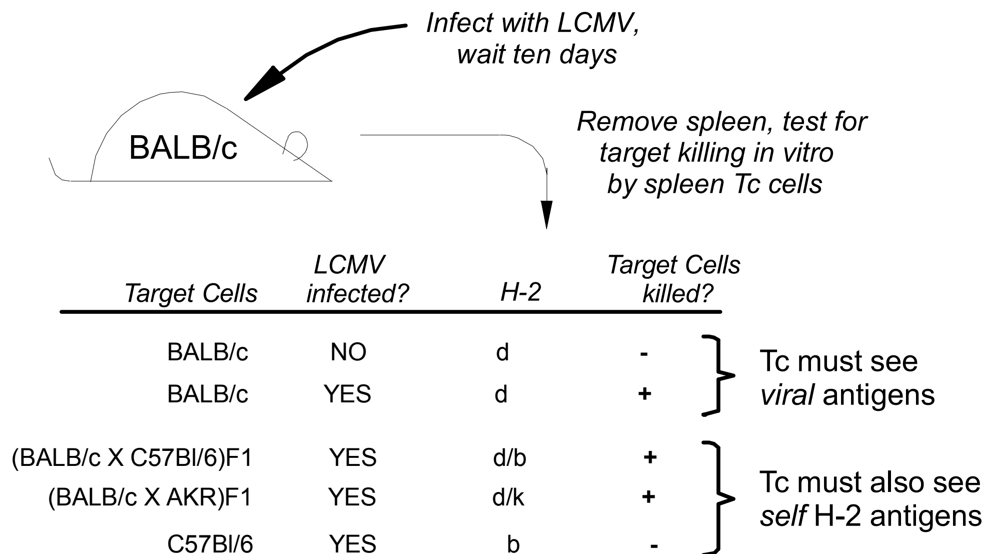
NK cells can also kill antibody-coated targets by a different mechanism deriving from the fact that NK cells bear Fc receptors, resulting in a mode of killing which is known as ADCC (Figure 12-3). Macrophages and neutrophils (as indicated in the figure) also bear Fc receptors and may also mediate this type of target cell killing. ADCC is a striking example of the complex relationships between innate and adaptive immunity, which in this case results in target cells being killed in a specific fashion by cells which are inherently non-specific.

In the remainder of this chapter we will discuss in more detail the basis of Tc-mediated target cell lysis, the first of these three mechanisms, illustrated in Figure 12-1.

ASSOCIATED RECOGNITION IN T-CELL KILLING: *MHC-RESTRICTED RECOGNITION*

One of the most striking and important findings to emerge from half a century of research in cellular immunology is that **cytotoxic T-cells, in order to kill their targets, must simultaneously recognize not only a foreign antigen, but also self-MHC antigens**. We can illustrate this remarkable phenomenon with a diagram of the original experiment carried out by Doherty and Zinkernagel using mice infected with the LCM (lymphocytic choriomeningitis) virus, as shown in Figure 12-4.

Infected mice develop cell-mediated immunity which can be detected by an *in vitro* assay. Spleen cells from such immune mice can kill LCM-infected target cells in culture; they are clearly LCM-specific, since they will not kill uninfected cells; this specificity is illustrated by the results shown in the first two lines of results in the figure.



Target Cells	LCMV infected?	H-2	Target Cells killed?
BALB/c	NO	d	-
BALB/c	YES	d	+
(BALB/c X C57Bl/6)F1	YES	d/b	+
(BALB/c X AKR)F1	YES	d/k	+
C57Bl/6	YES	b	-

MHC-RESTRICTED RECOGNITION BY KILLER T-CELLS

Figure 12-4

But the specificity for LCMV obviously doesn't tell the whole story, since the immune BALB/c spleen cells will *not* kill cells derived from a different strain of mice, even if they are LCMV-infected – in this example they will kill only BALB/c or F1 targets (shown in lines 3-5 in the figure). This was the first demonstration that *cytotoxic T-cells, in order to kill their targets, must recognize LCMV antigens on the target cell in association with "self" MHC molecules.* More precisely, they must recognize the viral antigens associated with self *Class I molecules* of the MHC complex (namely *K, D or L*-molecules of mouse H-2, or *A, B or C* molecules of human HLA). This phenomenon is known as **MHC-Restriction** or **MHC-Associated Recognition**.

CELL COOPERATION IN CELL MEDIATED IMMUNITY

MHC-associated recognition in target cell killing is part of a more general phenomenon which reflects the mechanism by which the T-cell receptors of all T-cells recognize their specific antigens. The diagram on the next page (Figure 12-5) illustrates the central features of the induction of CMI, including MHC-associated recognition.

The induction of CMI requires the cooperation of **three cell types**, an **effector cell** (in this case a cytotoxic T-cell, TCcell), a **helper** cell (TH1) and an **accessory** cell (*e.g.* dendritic cell, macrophage or other antigen-presenting cell). **Antigen** (a viral peptide in this example) must also participate, illustrated by the solid triangles. The final result of the cell/cell interactions and differentiation events described below is the killing of a virus-infected **target cell**.

"ASSOCIATED RECOGNITION" IN CELL-MEDIATED IMMUNITY

Cell cooperation in the generation of effector T-cells directed against viral antigens.

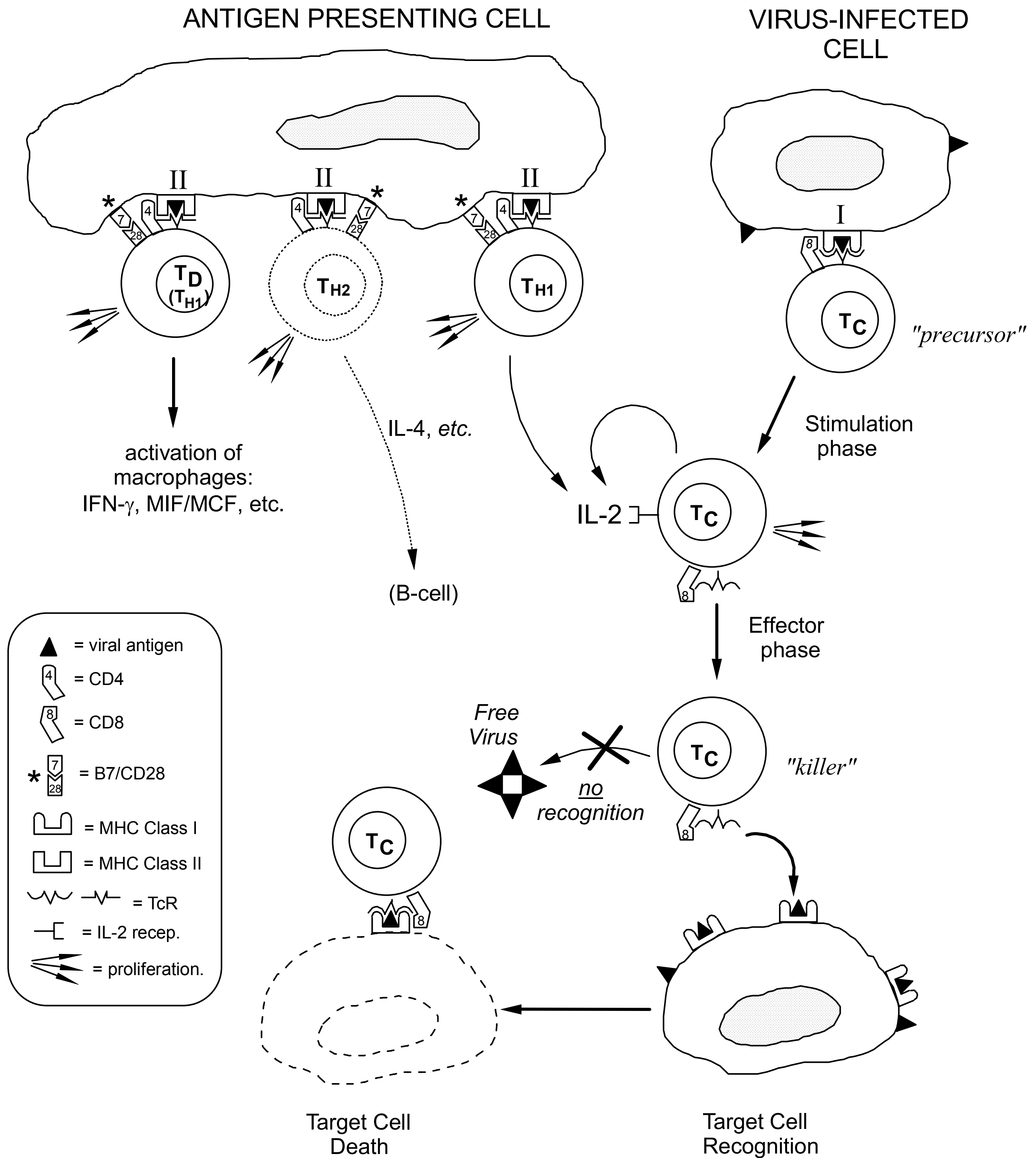


Figure 12-5

Some key features of these interactions are as follows:

- The *antigen-presenting cell* ("*accessory cell")* must have acquired the foreign antigen (an LCMV peptide) by capturing it from the surrounding tissue fluid (by phagocytosis or pinocytosis) and expresses it on its cell surface in association with *MHC Class II molecules*.
- A helper T-cell, the T_{H1} cell, clonally precommitted to an LCMV antigen, binds to the *accessory* cell. Its TCRs must recognize LCMV antigens *together with MHC Class II molecules*, and this interaction is therefore described as being *Class II restricted*. The T_{H1} cell bound in this manner now also recognizes and binds the B7 molecules on the antigen-presenting cell with its own CD28 membrane molecules. Specific binding of *both* these T-cell membrane receptors (TCR and CD28) results in this T_H-cell being triggered to secrete the cytokine IL-2 (Interleukin-2). among others.
- Meanwhile, a potential *target cell* must have been *infected* with the virus, and expresses LCMV antigens in association with *MHC Class I molecules*.
- A *clonally precommitted* T_C *cell* recognizes LCMV antigen on the target cell by virtue of its antigen-specific receptors (TCR), but in this case *complexed with MHC Class I*; this interaction is therefore described as being *Class I-restricted*. As a result of this interaction, the T_C cell is stimulated to express receptors on its surface for the cytokine IL-2.
- IL-2 produced by the T_{H1} cell is bound by the nearby T_C cell (now expressing IL-2 receptors), which is thereby triggered into its final stage of differentiation, becoming an effective killer cell. (This T_C cell, on being triggered to express the IL-2 receptor, may also *itself* secrete some IL-2 which can bind to and activate its own receptors in an *autocrine* process). This T_C can then bind to an LCMV-infected target cell, *recognizing LCMV antigens together with Class I molecules*, and causes the lysis of the target. This represents a second "*Class I restricted"* interaction.
- *The 2-Signal Principle*. These phenomena illustrate the general principle that *all T-cells require two distinct molecular signals in order to be triggered to differentiate and proliferate*, and ultimately carry out their effector roles in immune responses; one signal is antigen-specific, the other is not. In the case of T_H cells, the first signal is produced by occupancy of their TcRs (when they bind to their specific antigen together with MHC Class II on the APC), and the second ("costimulatory") signal is given by CD28 binding to B7. In the case of T_C cells, the first signal is provided by occupancy of their TcRs by antigen/MHC Class I, and the second signal is given by IL-2 binding to the newly expressed IL-2-receptors. We will see later that *B-cells also require two distinct molecular signals for activation*.

If the T_H cell in the above example were a T_{H2}-type cell (illustrated in dotted lines at the top of Figure 12-5), its interaction with the Class II-presented antigen would result in this cell's producing IL-4 (among other cytokines), which can stimulate a B-cell to differentiate into an antibody-producing cell; T-cell/B-cell cooperation in humoral responses will be discussed in Chapter 15.

Also illustrated in this diagram is the interaction between the accessory cell and *another* T_{H1} cell (labeled T_D, in the upper left), but in this case the T-cell produces macrophage chemotactic factors (MCF/MIF) as well as macrophage-activating factors such as IFN-γ. This interaction similarly requires recognition of antigen together with self Class II (*i.e.* it is *Class II restricted*). The production of this mix of cytokines leads to other manifestations of

CMI such as the tuberculin skin reaction (a classic example of DTH), and is an important part of the mechanism of resistance to infection by intracellular bacteria such as Listeria (discussed below).

IMPORTANCE OF MHC-RESTRICTED RECOGNITION IN T-CELL TRIGGERING

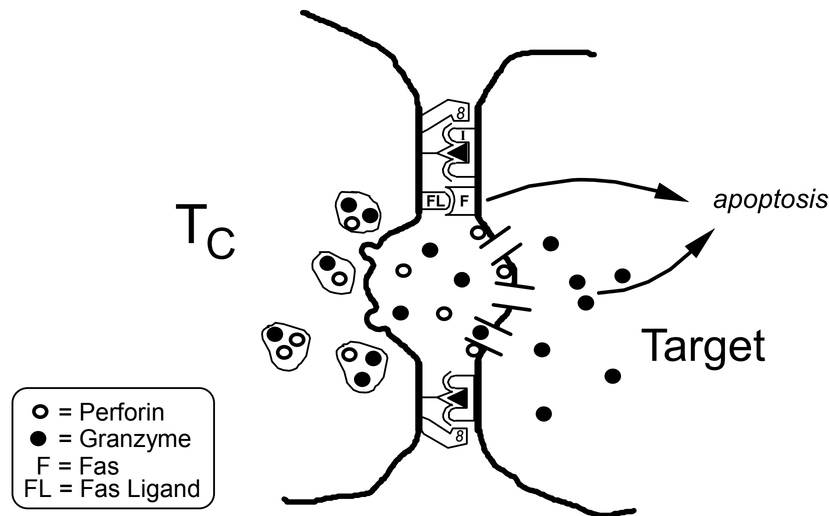
T-helper cells, which are clonally precommitted, antigen-specific cells, recognize their cognate antigens *only* in association with MHC Class II molecules, and therefore are triggered to differentiate and produce relevant lymphokines only when they are presented with antigen by an authentic antigen-processing cell (macrophage, dendritic cell, etc.). The importance of this phenomenon may lie in controlling the location and nature of such interactions, since *not all cells bear Class II antigens*. It would be pointless, for instance, for a T_H-cell to attempt cooperation with a muscle cell, even if there were antigen on its surface, since the muscle cell would be incapable of triggering the T-cell with the required "costimulatory" signals (*e.g.* by expressing B7). Such an interaction cannot be attempted, however, since muscle cells lack Class II antigens.

T-cell killing, on the other hand, is restricted with respect to *Class I* antigens, which are present on *all* nucleated cells. The significance of this restriction of T_C responses can be readily understood, since one of its effects is to assure that T_C cells can only be triggered by membrane-bound antigens. It would be counterproductive to permit T_C cells to be triggered by *soluble* antigen, say, in the example of an LCMV infection. *First*, inactivation of free virus can be readily accomplished by antibody, and does not require the participation of T_C cells; in fact, a Tc cell cannot generally do any harm to a free viral particle. *Second*, such stimulation would divert T_C cells from their critical role in killing virus-infected cells, which cannot be effectively dealt with by circulating antibody.

Although MHC-restricted recognition was first described in mice, precisely the same phenomenon regulates immune responsiveness in humans. In this case, of course, Class I antigens of the HLA complex are the A, B and C antigens, and Class II is represented by the DP, DQ and DR antigens (sometimes also loosely but incorrectly referred to as Ia antigens, by analogy with mouse H-2).

CYTOTOXIC T-CELLS KILL BY INDUCING APOPTOSIS

Cytotoxic T-cells kill their targets by inducing apoptosis (programmed cell death) using at least two mechanisms, as illustrated in Figure 12-6. As we have already seen, the TC cell binds to the target via its T-cell receptors, which recognizes MHC-Class I-associated antigenic peptide. (The participation of CD8 is also illustrated here, but not a variety of other membrane adhesion molecules which are also known to be important.) Subsequent binding of the target cell's membrane-associated *Fas* molecule by the T-cell's *Fas-ligand* is one well-known mechanism for inducing apoptosis. In addition, the T-cell releases the contents of specialized granules which contain two proteins, *perforin* and *granzyme*. The perforin inserts itself into the target cell membrane and forms large pores. The granzyme then enters the target cell through these pores and initiates a cascade of signaling events which ends by inducing the cell to undergo apoptosis. [NOTE: Under experimental conditions many target cells can be killed by perforin alone, in a manner similar to the effect of the membrane-attack complex (MAC) of complement. This simple lytic mechanism, however, appears not to be a significant factor in T-cell killing *in vivo*.]



APOPTOSIS-DEPENDENT KILLING OF TARGET CELLS
BY CYTOTOXIC T-CELLS

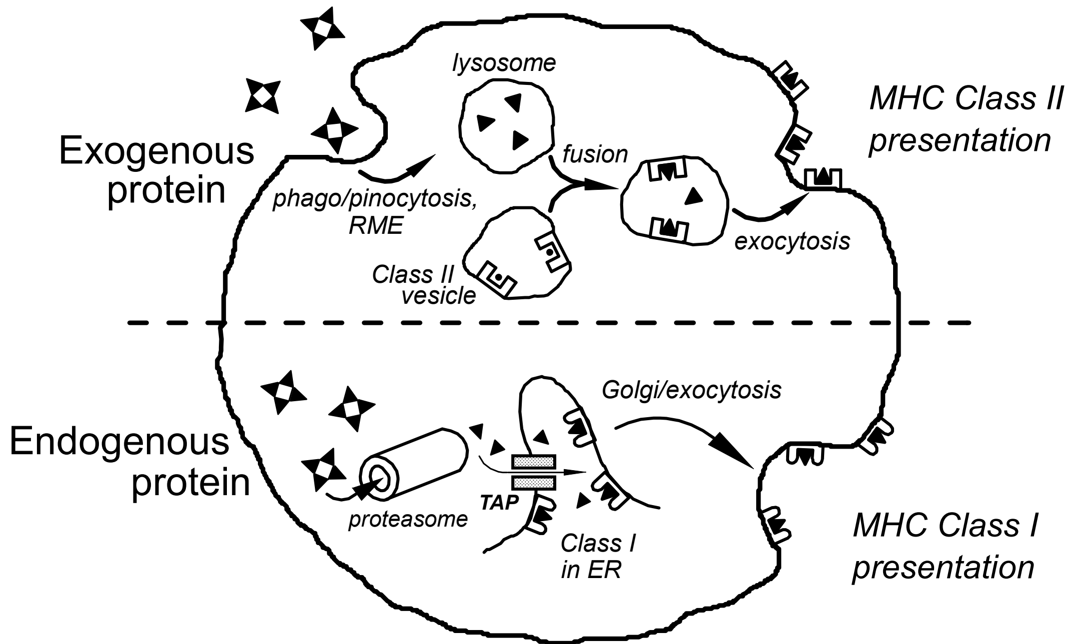
Figure 12-6

CLASS I AND CLASS II ANTIGEN ASSOCIATION REPRESENT TWO DISTINCT INTRACELLULAR PATHWAYS FOR ANTIGEN PROCESSING

As discussed above, antigens (or, more precisely, antigen-derived peptides) can be associated with either Class I or Class II MHC molecules; these two forms of MHC association result in *different modes of T-cell recognition* and *different biological consequences*. One must then ask the question, **how does a cell decide whether to express a particular peptide in association with either Class I or Class II MHC molecules**?

The general rule to remember is that **endogenous peptides (*i.e.* those derived from proteins synthesized within the cell) become associated with Class I MHC molecules**, **whereas exogenous peptides (those taken up by phagocytosis or pinocytosis) become associated with Class II MHC molecules**. This implies that separate intracellular pathways exist for the expression of the two classes of MHC molecules, and these are illustrated schematically in Figure 12-7.

Exogenous proteins may enter an antigen-presenting cell either by phagocytosis or some other form of endocytosis, and are incorporated into lysosomal vesicles in which they are degraded to small peptides. These lysosomes fuse with other vesicles which have emigrated from the ER and contain newly synthesized Class II molecules, and in the acidic environment created by the lysosome the Class II molecules can bind the peptides in their specialized groove. The small black dot shown in the “new” Class II molecules indicates the presence of the so-called “CLIP” peptide, part of a system responsible for preventing the Class II molecule from accepting a peptide before fusion of its vesicle with a lysosome, as well as for directing these vesicles toward the lysosomal compartment.



SEPARATE INTRACELLULAR PATHWAYS FOR CLASS I AND CLASS II PRESENTATION OF PEPTIDES

Figure 12-7

Endogenous proteins which are present in the cytoplasm (*i.e.* those synthesized within the cell itself) may encounter a "proteasome" which cleaves them into smaller peptides and feeds these peptides to the "TAP" transporter which pumps them into the lumen of the endoplasmic reticulum. Once in the ER, these endogenous peptides can be bound by newly synthesized MHC Class I molecules, and these complexes are transported via the normal secretory pathway in vesicles which eventually fuse with the plasma membrane. It's important to recognize that while our focus in this discussion has been how *viral* peptides come to be exposed on the surface of an infected cell, this process encompasses *all* endogenous peptides, not just those of viral origin. Thus, the MHC Class I molecules on the surface of any cell will present a sampling of *all* the peptides produced inside that cell, normal or viral.

Thus, in cells which *lack* Class II molecules (non-antigen-presenting cells), only endogenous peptides are displayed on its surface, and only in association with Class I MHC molecules (remember that virtually *all* nucleated cells bear Class I). In those cells which express Class II as well as Class I (*e.g.* macrophages, dendritic cells and B-cells), *endogenous* peptides are associated only with Class I (and can serve as targets for T-cell mediated killing), while *exogenous* peptides are associated only with Class II (and can serve to "present" antigen to helper T-cells). (NOTE: While this separation of Class I and Class II pathways generally holds and is important, there are intracellular mechanisms, not discussed here, which can cause some exogenous peptides to become associated with MHC Class I, and *vice versa*.)

One other important feature of MHC-peptide interactions should be kept in mind, namely that *the peptide-binding grooves of MHC molecules, whether Class I or Class II, are already occupied with a peptide at the time they appear on the cell surface.* Peptides become associated with MHC molecules during the process of assembly of these molecules, and do

not generally associate with pre-existing MHC molecules on a cell membrane (except under experimental or otherwise unusual conditions). As an illustration of this principle, mutant cells which *lack* a functional TAP transporter system express few if any Class I molecules on their surface, resulting in one form of a disease known as “Bare Lymphocyte” syndrome. In the absence of suitable peptides to occupy their binding grooves, MHC molecules are degraded and never appear on the cell surface.

It is also important to recognize that antigenic peptides from the same protein or viral particle may be associated with *both* Class I and Class II molecules on the same cell. A macrophage may, for example, be infected with LCMV; it will therefore express Class I-associated LCMV peptides (as does any infected cell), in addition to Class II-associated peptides (from phagocytosed virus particles). In this case the antigen-presenting accessory cell may itself be a target for cell-mediated killing.

SUPERANTIGENS: PATHOLOGICAL TRIGGERING OF T-CELLS

The mechanism which we have described for T-cell MHC-restricted antigen recognition and triggering has provided a variety of opportunities for subversion of the immune system by pathogenic microorganisms. An example of this is the production by various microbes of SUPERANTIGENS, one of which is the staphylococcal protein *Toxic Shock Syndrome Toxin-1* (TSST-1) whose mode of action is illustrated in Figure 12-8.

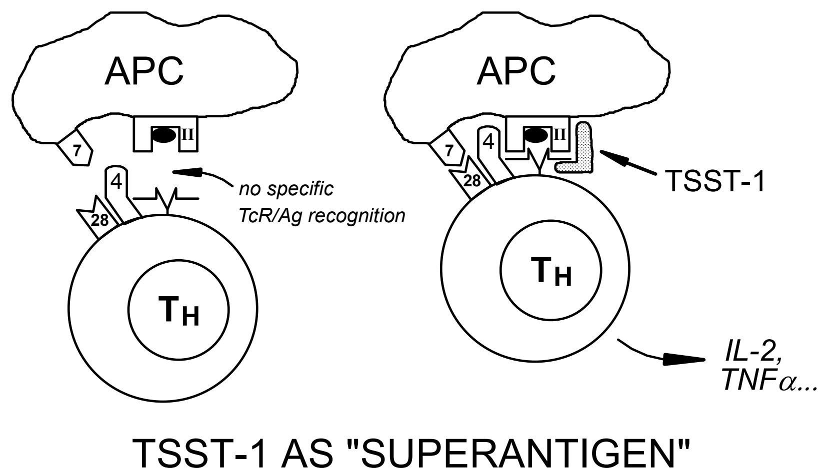


Figure 12-8

“Superantigens” have the unique property of being able to simultaneously bind to *both* the T-cell receptor and MHC Class II, thus effectively cross-linking a $CD4^+$ T-cell with a Class II-bearing cell (*e.g.* an antigen-presenting dendritic cell or macrophage). This cross-linking takes place *regardless* of whether the TcR has any specificity for the particular ClassII-bound peptide presented by the APC, but nevertheless results in activation of the T-cell and production of high levels of lymphokines. Any particular superantigen will be capable of binding anywhere from 2% to 20% of all TcRs (depending on which Vβ domain is expressed in the TCR), thus triggering a significant proportion of all the body’s T-cells. (Remember that normal immune responses engage only a very small proportion of T-cells, a small fraction of one percent, and that lymphokines are normally present at substantial concentrations only locally, very close to the site where they are produced.) The resulting high systemic level of lymphokines can be extremely toxic and is the cause of Toxic Shock

Syndrome (as in the illustrated example) and other diseases. Other microbial superantigens include several of the staphylococcal enterotoxins as well as some virally encoded membrane proteins.

ALLOGRAFT REJECTION: AN "EXCEPTION" TO THE RULE??

The above examples and discussion illustrate the important general rule that T-cells must recognize *self* MHC molecules in order to be triggered to either provide "help" (in the case of T_H) or to kill target cells(for T_C). If such recognition of *self* Class I MHC molecules were a simple absolute requirement for CMI, we should fail to see graft rejection between different strains of mice or between most unrelated humans, which in either case may not share any identical MHC molecules. This, of course, is not the case, and rejection of allografts takes place primarily as a result of the recognition of foreign Class I molecules in the *absence* of self recognition; it could therefore be regarded as an "exception" to this rule. The explanation of this dilemma resides in the complex pattern of differentiation which T-cells undergo within the thymus, which we will discuss in some detail when we cover the phenomenon of TOLERANCE in Chapter 18; this process involves both positive and negative selection of T-cells based on the nature of their T-cell antigen receptors, one result of which is their *ability to directly recognize many foreign MHC molecules*.

It is also worth keeping in mind, however, that *allografts are really experimental artifacts*; transfer of tissues or organs between individuals (at least in higher organisms such as vertebrates) does not normally take place in nature, and cell-mediated immunity has not evolved for the purpose of dealing with such transplants.

IMMUNE RESPONSE ("Ir") GENES AND MHC RESTRICTION

An understanding of MHC-linked *immune response genes* has become increasingly important in understanding the basis of the *striking association of specific HLA haplotypes with a variety of human diseases,* particularly the large family of *rheumatoid diseases* involving autoimmunity and inflammation. Immune response ("Ir") genes were first described as genes which controlled the levels of antibody produced against certain T-dependent antigens. Some strains of mice, for instance, respond with high levels of antibody to the synthetic polypeptide TGAL, while mice of other strains respond poorly or not at all. The ability to make anti-TGAL antibody could be shown to be controlled by a single genetic locus, and was an early example of an Ir gene locus. Many other examples of Ir genes have since been described. *Most of these genes have been shown to be located in the Class II region of the MHC, and in fact are the Class II genes themselves.*

Why would MHC Class II genes behave as "immune response genes"? In what way could polymorphisms of Class II molecules encourage or prevent the generation of immune responses to specific antigens? As we've already seen, the generation of helper T-cells for any given antigen requires T-cell recognition of "processed" antigen coupled to a Class II molecule on the surface of an antigen presenting cell. It's been found that there exist certain allelic forms of Class II molecules (remember that they are *highly polymorphic*) which fortuitously cannot combine appropriately with a particular antigenic peptide; in such a case, *an organism which has only this ineffective version of Class II will not be able to mount an effective immune response to this particular peptide*. This phenomenon does *not* result in

general immunodeficiency, since it is *antigen-specific*, and responses to any other antigen will therefore be normal. A Class II allele which combines poorly with one antigen wlll be capable of combining very well with another.

IMMUNOLOGICAL DEFENSE AGAINST INTRACELLULAR BACTERIA: LISTERIA MODEL

T-cells can also participate in other modes of cell-mediated immunity which do *not* involve T_C-mediated target cell lysis. One example is the mechanism of resistance to intracellular parasites, this one *not* involving T-cell killing, is illustrated by the classic experiments of Mackaness using the bacterium *Listeria monocytogenes*, a gram-positive organism which is an animal and human pathogen. While this organism is effectively phagocytosed by macrophages, it continues growing inside these cells unless the macrophage has been activated by an antigen-specific T-cell. This is illustrated in Figure 12-9, below.

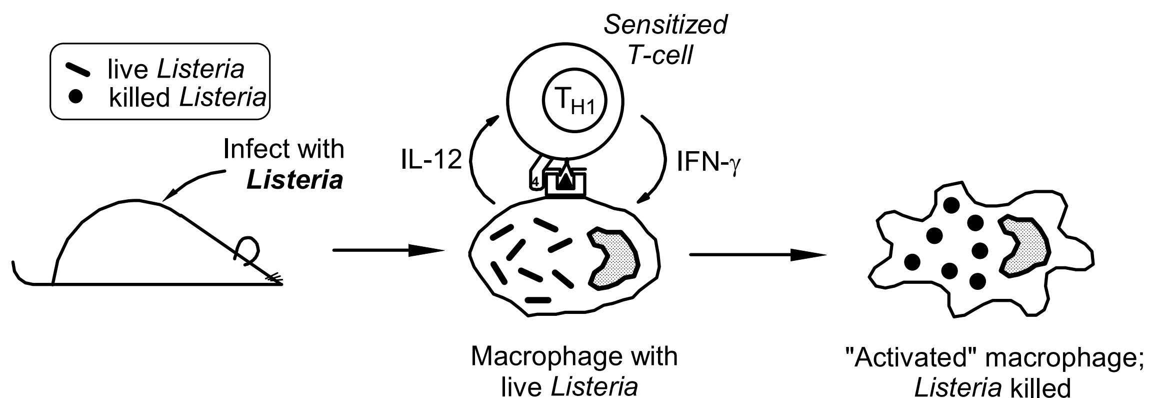


Figure 12-9

The T-cell (a T_{H1}-class cell) is activated by bacterial antigen through the mechanisms we have already discussed. This sensitized T-cell can then recognize the affected macrophage by its MHC Class II-associated Listeria antigens, and begins secreting interferon-gamma which "activates" the macrophage. The macrophage then becomes capable of killing the Listeria organisms it had already ingested. The macrophage, in turn, can produce IL-12 which further stimulates the T-cell to produce IFN-γ

> [NOTE: There are different mechanisms by which intracellular pathogens may survive inside mammalian cells. For example, Listeria enters and is able to multiply within the cell's cytoplasm itself, whereas Mycobacterium (see below) remains inside the cell's endocytic vesicles where it proliferates. The principles described here, of the requirement for macrophage activation by T-cells, nevertheless hold for both situations.]

Let's examine a hypothetical set of experiments, illustrated in Figure 12-10, which should clarify the distinct roles of T-cells (which are antigen-specific) and macrophages (which are *not* antigen-specific) in this disease model.

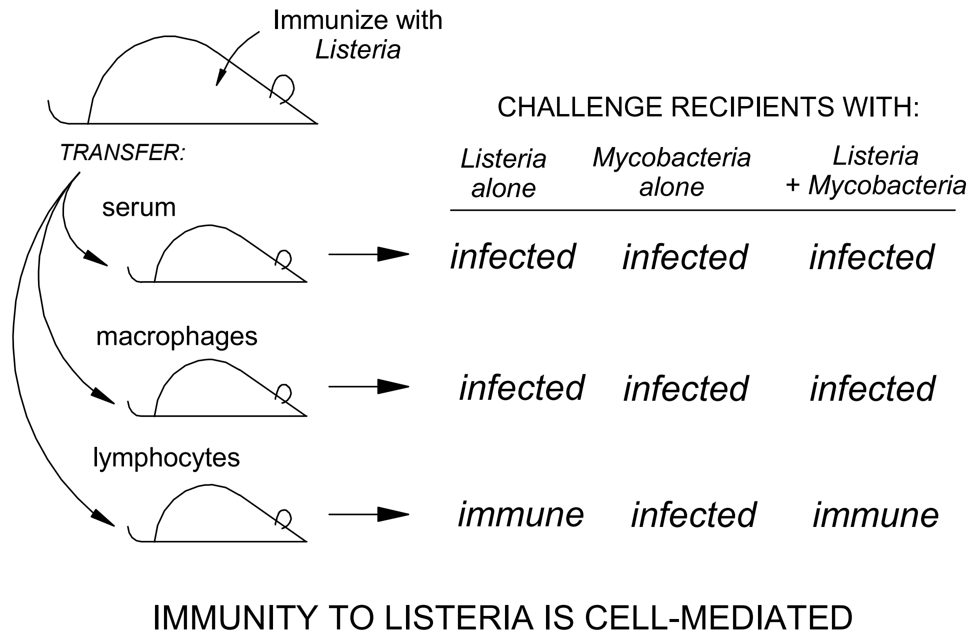


Figure 12-10

Immune resistance to *Listeria* can be adoptively transferred by using lymphocytes, but *not* by using macrophages, because *activated macrophages are relatively short-lived*.

A mouse adoptively immunized in this manner (by transer of immune lymphocytes) is resistant to a subsequent infection by Listeria, but not resistant to *Mycobacterium tuberculosis*, another intracellular bacterial parasite. However, if the recipient mouse is challenged with Mycobacterium *together* with Listeria, it will not develop an infection with either one. **The TH1 memory response in this example is specific only for Listeria antigens, but once the *macrophages* are activated, they are capable of non-specifically killing *either* organism**. These experiments in mice illustrate the principles by which *human* cell-mediated immunity can provide protection against tuberculosis and other intracellular bacteria.

CHAPTER 12, STUDY QUESTIONS:

1. Describe the original Doherty and Zinkernagel experiment (cytoxicity against LCM virus-infected target cells) which defined the phenomenon of MHC-RESTRICTED RECOGNITION.
2. Diagram the various CELL-TO-CELL INTERACTIONS which occur during the generation of a cytotoxic T-cell response. What are the cell surface molecules involved in each interaction?
3. With respect to MHC/PEPTIDE ANTIGEN PRESENTATION, describe the fate of a viral protein: a) following its production during a viral infection; b) following injection of the purified protein into a mouse.
4. Immune resistance to LISTERIA INFECTION and to a VIRAL INFECTION are both the result of cell-mediated immunity (CMI); in what ways do they differ?

CHAPTER 13
T-CELL/B-CELL COOPERATION IN HUMORAL IMMUNITY

See APPENDIX (12) PLAQUE-FORMING CELL ASSAY

Adoptive transfer experiments illustrate the use of antibodies to cell surface markers in distinguishing the roles of different cell types in the CELL/CELL COOPERATION required for humoral immune responses. While the THYMUS is *not* itself a site of immune reactivity, it is the source of T-CELLS ("helper" TH-cells) which are required to cooperate with B-CELLS (the precursors of antibody-forming cells) to generate antibody responses to THYMUS-DEPENDENT (TD) ANTIGENS, as well as those T-cells ("effector T-cells") responsible for CELL-MEDIATED IMMUNITY. Humoral responses to THYMUS-INDEPENDENT (TI) ANTIGENS, however, do not require T-cell help. While B-cells in mammals are produced in the bone-marrow, a distinct organ exists for their production in birds (the BURSA). One widely utilized animal model for immunodeficiency is the *athymic* or NUDE MOUSE. The congenital absence of a thymus in these mice results in the absence of T-cells, and consequently an almost complete lack of cell-mediated immunity and of humoral responses to TD antigens.

THE THYMUS IS REQUIRED FOR THE DEVELOPMENT OF IMMUNE RESPONSIVENESS

The thymus is an organ containing large numbers of lymphocytes, which in humans surrounds the top part of the heart. Until the 1950's nothing was known of its function, although its histology clearly made it part of the lymphoid system. Classical kinds of experiments to determine its function by surgical removal in adult animals gave no clear results – no physiological defects became apparent and the organ was apparently not missed. One unusual feature which has been recognized since ancient times is that the thymus starts out as a fairly large organ in very young animals (including humans) which continues to grow through early life, but then undergoes a process of *involution* or progressive degeneration and decrease in size, beginning at about the time of puberty. In older adult animals, the thymus is often little more than a small bit of connective tissue.

Our present understanding of thymic function developed only around 1960 through the experiments of J.F.A.P. Miller, who showed that the thymus was critical for the *development* of immune competence. For unrelated reasons, he had removed the thymus from mice within 24 hours of birth (*neonatal thymectomy*), and found that such mice grew poorly, suffered from a continuous series of viral and other infections, and often died before reaching adulthood, a condition known as *runting syndrome*. However, runting only developed if thymectomy was carried out within the first 24 hours of birth -- thymectomy in older mice had little or no effect, consistent with the results of the earlier experiments referred to above.

The cause of runting in these neonatally thymectomized mice was shown to be due to the fact that they are severely deficient in their ability to mount immune responses both to infectious agents (which accounts for their susceptibility to infections) and to experimental antigens.

This immunodeficiency includes both *humoral immunity* (poor antibody response to some, but not all, antigens), and cell-mediated immunity (lack of ability to reject skin grafts) and can be corrected by a *thymus transplant*. Thus, *while the thymus is not required for immune responses, it is necessary for the development of immune responsiveness*. We will see later that this is because T-cells are produced in the thymus and exported to the peripheral lymphoid tissues, and it is only in the periphery that they normally respond to antigen challenge.

SYNERGY IN IMMUNE RESPONSES: ANTIBODY FORMATION REQUIRES T-CELLS AND B-CELLS

One researcher who followed up on Miller's findings was Henry Claman, who in 1966 published the results of his studies on the role of the thymus in humoral antibody responses. He used an *adoptive transfer* system enabling him to *mix* cells from different sources to examine the antibody response to SRBC. He transferred thymus cells, bone marrow cells, or both, together with the antigen SRBC, into lethally irradiated recipients.

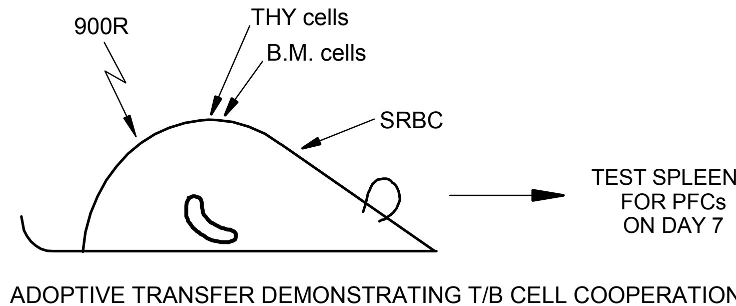


Figure 13-1

He measured the response *not* by assaying circulating antibody, but by removing the spleen and determining *the number of antibody-forming cells* it contained, using the newly-developed hemolytic **Plaque-Forming Cell (PFC) assay** (see APPENDIX 12). An example of his results is shown below:

Lymphoid Cells transferred	*PFC's per spleen*
none	20
thymus cells	50
bone marrow cells	60
thymus + bone marrow	550

These results show very little response with either thymus cells or bone marrow cells alone (a normal response to SRBC would yield 50,000 or more PFC's per spleen), but there was ten-fold higher response with *both* cell populations together than with either alone. This was the first evidence of *synergy*, or *cell cooperation* in immune responses. While Claman concluded that the humoral response required participation of cells from *both* thymus and bone marrow (which later became known as T-cells and B-cells respectively), he was unable to determine which of the two sources gave rise to the antibody-forming cells themselves.

B-CELLS BECOME ANTIBODY-FORMING CELLS -- T-CELLS ARE "HELPERS"

Mitchell and Miller were soon able to answer this question by developing a modified and more complex adoptive transfer system, and by using anti-H-2 antibodies to distinguish the different cell types. Instead of doing a short-term transfer into irradiated hosts, they injected bone marrow cells into mice that had previously been thymectomized as adults and lethally irradiated. The injected cells rescued the animals from radiation death by completely restoring their hemopoietic system, *except for T-cells*, which could not be regenerated in the absence of the thymus. After three weeks (to allow hemopoietic restoration to take place), the animals were immunologically equivalent to neonatally thymectomized animals -- they had the same immunological deficiency, which could be corrected by transplanting thymus tissue, or, as we will see below, by providing another source of competent thymus-derived cells. Such animals are known as **ATxBM** mice (for **A**dult **T**hymectomized, **B**one-**M**arrow restored).

The second improvement Mitchell and Miller were able to make was to use lymphocytes from the thoracic duct lymph (the major lymphatic vessel) instead of thymus cells. These **thoracic duct lymphocytes** (TDL's) contained more *mature* thymus-derived cells than thymus tissue itself, and resulted in a much higher adoptive response. Their adoptive transfer system is illustrated below:

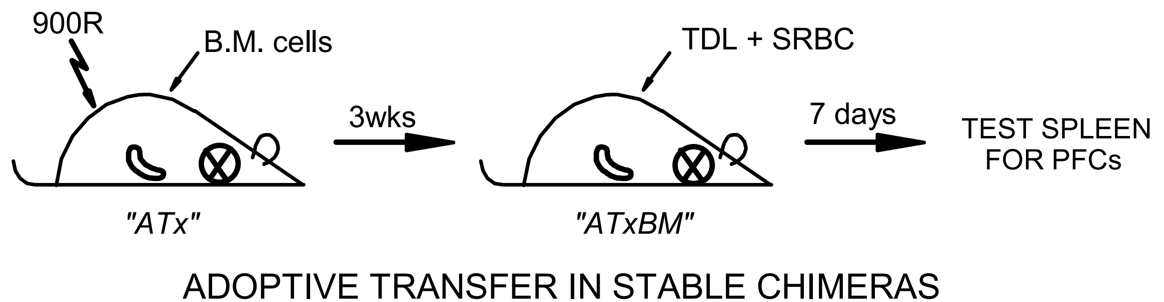


Figure 13-2

Using this new adoptive transfer system, and measuring the number of PFC's seven days after immunization, they obtained the kinds of results shown below:

Treatment of ATxBM Hosts	*Spleen PFC's*
SRBC alone	200
TDL + SRBC	11,000 (!!)

While the ATxBM animals responded almost not at all to SRBC, addition of TDL's restored their response to levels comparable (although not quite equal) to those of normal, intact animals.

The third advantage of this system turned out to be the fact that they could get this high level of responsiveness using cells derived from *genetically different donors*. They constructed an adoptive transfer with the following MHC types:

	Strain	*H-2 Haplotype*
ATxBM HOST	C57Bl/6	($H\text{-}2^b$)
BONE MARROW	(C57Bl/6 X BALB/c)F1	($H\text{-}2^{b/d}$)
TDL	C57Bl/6	($H\text{-}2^b$)

Note that in this particular combination *all* cells bear H-2 antigens of the $H\text{-}2^b$ haplotype, but *only* the bone-marrow cells (and therefore their progeny) bear $H\text{-}2^d$ antigens.

In order to exploit the MHC difference between the two cell populations, Mitchell and Miller added one additional step to the assay procedure: after removing the spleen cells for assay, but before placing them in the PFC assay system, they treated the cells with anti-H-2 antisera plus complement. This treatment killed any cells bearing those H-2 antigens recognized by the antibody, and such cells would therefore *not* be seen in the PFC assay. They used two antisera, one detecting $H\text{-}2^b$, the other $H\text{-}2^d$. If the antibody-forming cells are derived from bone-marrow, they should be killed by *both* antisera; if they are derived from TDL, they should be killed *only* by anti-$H\text{-}2^b$. The results they actually obtained are illustrated below:

Treatment of cells	*PFC per spleen*
none	11,000
anti-$H\text{-}2^b$	550
anti-$H\text{-}2^d$	450

These results show that these antibody forming cells can be killed by *both* anti-H-2 antisera, and therefore the antibody-forming cells must have been derived from the bone marrow, and not from the TDL's. Thus, they demonstrated that *while both "B cells" (bone-marrow derived) and "T cells" (thymus-derived) are required to generate a humoral response to SRBC, only the B-cells develop into antibody-forming cells, while the T-cells perform a "helper" function*. The nature of this "helper" function will be discussed in Chapter 15.

THYMUS-DEPENDENT VERSUS THYMUS-INDEPENDENT ANTIGENS

Not *all* humoral responses are absent in neonatally thymectomized animals; T-cell deprived animals can respond quite well to a number of antigens, which is the basis for distinguishing between *thymus-dependen*t and *thymus-independent* antigens.

Thymus-dependent (TD) antigens generally include most protein antigens and most cell surface antigens (which are commonly glycoproteins). Such molecules are generally capable of inducing not only primary (IgM) responses, but also *memory responses, i.e*. class switching to IgG, IgA and IgE isotypes.

Thymus-independent (TI) antigens include polysaccharides with highly repetitive epitopes; since many antigens in bacterial cell walls (and other microorganisms) bear

such structures, this is clearly a biologically significant category. These antigens commonly induce *only primary (IgM) responses* with little or no memory or class switching. (See Chapter 15 for more detail on TI antigens.)

THYMUS AND BURSA IN BIRDS: SEPARATE PRIMARY LYMPHOID ORGANS FOR T CELLS AND B CELLS

In addition to having a thymus analogous in structure and function to that of mammals, members of the Class Aves (birds) have another lymphoid organ called the **Bursa of Fabricius**, located near the cloaca. Removal of the *embryonic thymus* before hatching results in chickens with no cellular responsiveness (they cannot reject skin grafts), and deficient humoral responses to many, but not all antigens; this is similar to what we have seen following neonatal thymectomy in mice, and in congenitally athymic ("nude") mice (see below). Removal of the *embryonic Bursa*, on the other hand, results in a lack of ability to make *any* humoral response, while the ability to reject grafts (and carry out other cell-mediated immune responses) is unaffected.

Both the Thymus and Bursa are considered to be **Central Lymphoid Organs** in birds; T-cells mature in the thymus and B-cells in the Bursa, although they actually carry out their immune responses in other (peripheral) organs. There is no distinct organ in mammals homologous to the Bursa of birds, and mammalian B-cells are produced largely within the bone marrow itself.

[It is interesting to note that the chicken was the first system in which the dichotomy between humoral and cellular responsiveness was made clear, and the term B-cell, in fact, was originally coined to refer to "Bursa-derived" cells.]

GENETIC THYMIC DEFICIENCY: THE "NUDE" MOUSE

Surgical removal of the neonatal thymus provided the earliest demonstration of that organ's importance in the development of the immune system. Soon afterwards, however, a genetic animal model of thymic insufficiency was discovered, namely mice homozygous for the recessive mutation *nu* (for "nude").

"Nude" mice, whose genotype is *nu/nu*, lack body hair (hence the name), and also *lack a functional thymus*. They show all the features of neonatally thymectomized mice, *i.e.*, "runting" and poor general health, inability to make humoral responses to many (but not all) antigens, and lack of ability to reject skin grafts and carry out other cell-mediated immune reactions. *They lack functional T-cells* in the blood and peripheral lymphoid tissues. Implanting a fetal thymus (*e.g*. under the kidney capsule) completely restores the normal level of T-cells as well as the ability to generate all normal immune responses.

The nude (nu/nu) mouse has been widely used for studies on T-cell function; it is a more reliable model than neonatally thymectomized animals, since this surgery is difficult to carry out and may result in a small amount of thymic tissue being left behind. *The nude mouse has also been a valuable tool for the study of heterologous tumors*, especially human tumors. In the absence of cell-mediated immunity, the foreign tumors are not rejected, as they would be in a normal animal, and they can be grown and transferred from mouse to mouse. Decades of

studies in tumor biology have relied on the ability of human tumor cells to grow in *nude* mice as a defining feature of their oncogenic potential. The human counterpart of the mouse *nu* gene has recently be shown to be the *whn* gene, and homozygosity for rare mutations at this locus result in a combination of hairlessness and immunodeficiency very similar to the condition seen in the *nu/nu* mouse

CHAPTER 13, STUDY QUESTIONS:

1. How was the role of the THYMUS in immunity first discovered?
2. Describe the experiments of Mitchell and Miller which defined the roles of T and B cells, and state succinctly what their conclusions were.
3. Define TI versus TD antigens. Give some examples of each which are of significance in people.
4. Why are there so many NUDE (*nu/nu*) MICE in the world?

CHAPTER 14

CELL SURFACE MARKERS OF T-CELLS, B-CELLS AND MACROPHAGES

An understanding of the distinct families of molecules present on different cells of the immune system provides the tools for distinguishing these cell types in both diagnosis and therapy, as well as understanding the molecular basis for many lymphoid cell functions. Diagnostic MARKERS for T-cells include the T-cell receptor (**TcR**) with its associated **CD3** signaling complex as well as **CD5** and the E-receptor (**CD2**). The presence of membrane immunoglobulin (**mIg**), which functions as antigen receptor, is diagnostic for B-cells. Complement receptors (**CR**) and Fc receptors (**FcR**) which can mediate opsonization, and **MHC Class II** molecules which are important in antigen presentation, are present on B-cells and macrophages (as well as dendritic cells). Two major classes of T-cells are distinguished by the presence of either **CD4** (on $\mathbf{T_H}$ and $\mathbf{T_{reg}}$) or **CD8** (on $\mathbf{T_C}$cell). T_H cells can be further subdivided into $\mathbf{T_{H1}}$,$\mathbf{T_{H2}}$ and $\mathbf{T_{H17}}$, which produce different combinations of cytokines and have distinct physiological roles.

We know that there are at least three major cell types required for immune responses, namely the T-cell, B-cell and macrophage. Conventional microscopy is only of limited usefulness in distinguishing these cells, and is of no use whatsoever in distinguishing various lymphocytes one from another (T-cells from B-cells, or different subpopulations of either).

Different classes of cells can readily be distinguished, however, by virtue of the fact that they express unique combinations of molecules in their membranes. Our knowledge of the different cell types involved in immune responses is a direct result of the development of reagents to distinguish these various cells by their **cell surface markers**. We will see that such markers include molecules distinguished either by antibodies directed against them, or else by their ability to bind various other molecules or cells.

SOME MARKERS ON HUMAN T-CELLS, B-CELLS, AND MACROPHAGES

	Ig	*TcR(&CD3)*	*FcR*	*CR*	MHC *ClassII*
T-cell	–	+	–	–	–
B-cell	+	–	+	+	+
Macrophage	–	–	+	+	+

Table 14-1

Ig. One defining characteristic of B-cells is the presence of *membrane immunoglobulin*, which T-cells lack. These immunoglobulins are IgM_S (μ_2L_2 “IgG-like” subunits) and IgD, they are anchored in the cell membrane through a specialized C-terminal domain of the heavy chain, and they function as the cells' antigen-specific receptors. Memory B-cells bear membrane-bound isotypes other than IgM, representing whatever Ig the cell will begin secreting upon antigenic stimulation.

TcR. The T-cell antigen receptor (TcR) is the principle defining marker of *all* T-cells. This molecule is used by the T-cell for specific recognition of MHC-associated peptide antigens, discussed in Chapter 12. Also associated with the TcR is a complex of proteins known as **CD3**, which participate in the transduction of an intracellular signal following TcR binding to its cognate MHC/antigen complex.

FcR. Various cells including B-cells, PMNs and macrophages have molecules on their surface known as **Fc-Receptors**, which are able of binding IgG through its Fc region. Aggregated or antigen-bound IgG binds much more strongly to these receptors than free, soluble IgG, so that B-cells or macrophages taken directly from an animal will normally have little or no Fc-bound Ig on their surface. The function of Fc-receptors on macrophages is easy to understand in that they mediate **opsonization**. The function of these receptors on B-cells is less clear, and most likely is involved in regulating humoral responses. When determining if a cell bears surface Ig (to identify B-cells), one must always be careful to account for the possibility of passive Fc-binding (especially to monocytes and/or granulocytes), which may give misleading results.

CR. B-cells and macrophages can also bind various complement components, either in free form or as part of an immune complex, by their *complement receptors* (CR), of which there are at least five known forms. One form of CR known as "CR1" binds C3b and C4b, and is expressed on macrophages, PMNs, B-cells and erythrocytes. As is the case for Fc receptors, the presence of CR on macrophages clearly enhances *opsonization* (mainly through binding of C3b), while the significance of CR1 on B-cells is less clear. The ability of CR1 to bind C4b is the basis for its role in the normal clearance of soluble immune complexes by *erythrocytes* (see Chapter 5). T-cells lack complement receptors.

MHC Class II. Class II MHC molecules in humans include DP, DQ and DR. (In the mouse they are known as *Ia* molecules, or **I**-region **a**ntigens). Unlike MHC Class I, Class II molecules are *not* expressed on all cells; they are present on B-cells, macrophages and other antigen-presenting cells, but *not* on cells of most non-lymphoid tissues. They are not expressed on "resting" T-cells, but may be induced at low levels when the T-cell is activated. T-cell receptors on "helper"-type ($CD4^+$) T-cells recognize specific antigenic peptides *only* when the peptides are associated with MHC Class II molecules on the surface of an antigen-presenting cell (discussed in Chapters 12 and 15).

ANTIBODIES WHICH DISTINGUISH HUMAN T-CELL SUBPOPULATIONS

T-cells consist of a variety of subpopulations, each of which can carry out one or more specific immune functions. One important way in which these subpopulations can be distinguished is by the use of the two cell surface markers *CD4 and CD8*.

Distinct Classes of T-Cells

	Class of Cells	Cell Function
T_H (**$CD4^+$**)	**T_{H1}**	Helper cell for CMI, stimulates T_C maturation and activity; recruits macrophages in generating DTH response.
	T_{H2}	Classical "T-helper"; stimulates B-cell proliferation and differentiation.
	T_{H17}	T cell" with pro-inflammatory role, facilitates activity of other cells, including Tc, NK and macrophages
	T_{reg}	"*Regulatory* T-cell"; suppresses development or execution of both B-cell and T-cell immune responses
T_C (**$CD8^+$**)		"*Cytotoxic* T-cell"; kills antigen-bearing target cells; acts as effector cell in graft rejection and other cell-mediated immune responses

Table 14-2

Mature T-cells express only one or the other of these two cell surface antigens, whose molecular roles in cell cooperation we have discussed in Chapter 12. One class of T-cells is $CD4^+CD8^-$ and includes the "helper" or T_H cells which may be involved in cooperative interactions with either B-cells (T_{H2}) or with other T-cells (T_{H1}), or play other pro-inflammatory roles (T_{H17}). T_{reg} or "regulatory" T-cells, which play an important role in tolerance (see Chapter 18), also express this $CD4^+$phenotype. The second major category of T-cells defined by these two markers ($CD4^-CD8^+$) encompasses the "cytotoxic" or T_C cells which have important effector functions in graft rejection and other cell-mediated immune reactions.

The helper class of T-cells can be divided into three sub-categories, based not on cell surface markers but on the lymphokines they produce and the kinds of immune reactions in which they participate. T_{H2} cells are the classical T_H "helper" cells originally defined by their role in providing B-cell "help" in humoral immune responses. T_{H1} cells, on the other hand, are

"inflammatory" T-cells which provide "help" to other T-cells (*e.g*. in development of graft rejection) or directly promote inflammatory reactions by their actions on macrophages and PMN's (*e.g.* in skin DTH reactions). The roles of T_{H1} and T_{H2} cells are discussed in more detail in Chapters 12 and15. A more recently defined class of $CD4^{+}CD8^{-}$ T_H cell is the "T_{H17}" cell, which produces a mix of cytokines including IL-17. This "pro-inflammatory" cell stimulates the differentiation and activity of a variety of other cells important in protective immune resoponses, including NK cells, PMNs and macrophages.

A wide variety of antibodies have been developed which distinguish human T-cell subpopulations. These have been the result of the development of HYBRIDOMA technology (see APPENDIX 13), and a few of the commercially available antibodies are listed below:

"CD" Name	Anti-human monoclonal Antibodies	Distribution on Human Cells
CD2	(OKT-11, Leu-5)	All T ("E-receptor")
CD3*	(OKT-3, Leu-4)	All T
CD4*	(OKT-4, Leu-3)	T_H ,T_{reg} (+ macrophage)
CD5	(OKT-1, Leu-1)	All T
CD8*	(OKT-8, Leu-2)	T_C

****You should be familiar with the distribution of these three markers and their biological significance.***

Table 14-3

The **CD4 and CD8** molecules (and the antibodies directed against them) are of particular importance in clinical medicine. The loss of CD4-bearing T-cells, for example, is associated with disease progression in AIDS, and determination of the CD4:CD8 ratio is a standard tool in the evaluation of HIV-positive patients. CD4 itself also plays an important role in HIV infection, as it is the major receptor by which the virus enters those cells which it infects (T-cells and macrophages).

The *monoclonal antibodies* to human lymphocyte antigens outlined above have been tremendously useful in diagnostic medicine for evaluating the immune status of patients. Use of these reagents can yield information about the relative abundance of different functional T-cell subsets, which is much more useful than knowing only the total number of lymphocytes, or even the relative number of T-cells and B-cells. In addition, such reagents can be used therapeutically for specific removal of a particular T-cell subset, as, for example, the use of anti-CD4 to inhibit T_H cell function in autoimmune disease.

A note on nomenclature: The confusing naming systems historically associated with many cell surface markers in different species over the years has been made more rational by the adoption of a standard nomenclature system. If cell surface antigens in different species (which often are assigned unrelated names as they are discovered) are determined to be homologous by molecular characterization, they are assigned a standard name in the **CD series** ("**C**omplex of **D**ifferentiation"). Thus, CD3 molecules in human, mouse, rat, *etc* all have similar sequences and structures. Despite such structural homology, however, identical CD antigens *do not necessarily have the same tissue distribution or function* in different species. For example, CD5 in humans is present on *all* T-cells, whereas CD5 in mice (originally called Ly-1) is present only on the *helper* subpopulation of T-cells. This is just one example of many differences in the patterns of differentiation of T-cells between humans and other mammalian species.

FUNCTIONAL MARKERS FOR LYMPHOCYTE SUBSETS: MITOGENS AND OTHERS

While the presence of one or another cell surface antigen has long been the most widely used method for distinguishing different lymphoid cells, other approaches have also been used, for example the use of **mitogens** to activate different classes of lymphocytes.

A mitogen is any substance which stimulates a non-cycling cell to undergo mitosis, and many mitogens are members of a large group of carbohydrate-binding molecules known as *lectins*. **Concanavalin A (ConA)** is one such lectin, which selectively stimulates T-cells to divide in both humans and rodents; the proliferative response of a lymphocyte population to ConA can therefore be used as a crude measure of the proportion of T-cells it contains. Another lectin, **Pokeweed Mitogen (PWM),** is selective for rodent B-cells and can therefore be used experimentally as a measure of the proportion of B-cells in a population.

Other functional characteristics can also be used to distinguish various populations of lymphocytes. The ability to transfer a *DTH response*, for instance, is the defining characteristic of T_D (or T_{DTH}) cells (which are mostly TH1), and can be used to detect their presence. As another example, macrophages/monocytes (and granulocytes) can be readily distinguished from lymphocytes by virtue of their ability to *adhere* tightly to glass or plastic surfaces, as well as by their relative resistance to the toxic effects of *ionizing radiation*. For example, adherence can be used to selectively remove monocytes from a mixed population of blood leukocytes, leaving behind mostly lymphocytes. Exposing blood leukocytes to sufficient doses of ionizing radiation, on the other hand, will selectively kill lymphocytes, and the surviving cells will be mostly monocytes and granulocytes.

CHAPTER 14, STUDY QUESTIONS:

1. Describe those cell surface markers which characterize and distinguish B-CELLS, T-CELLS AND MACROPHAGES.
2. What functional roles do FcR and CR play in the immune system?
3. Describe the distinct roles of T_{H1} and T_{H2} cells in immune responses. Which cell markers do they share? Which markers may be used to distinguish them?

CHAPTER 15
CELL INTERACTIONS IN HUMORAL RESPONSES: THE CARRIER EFFECT

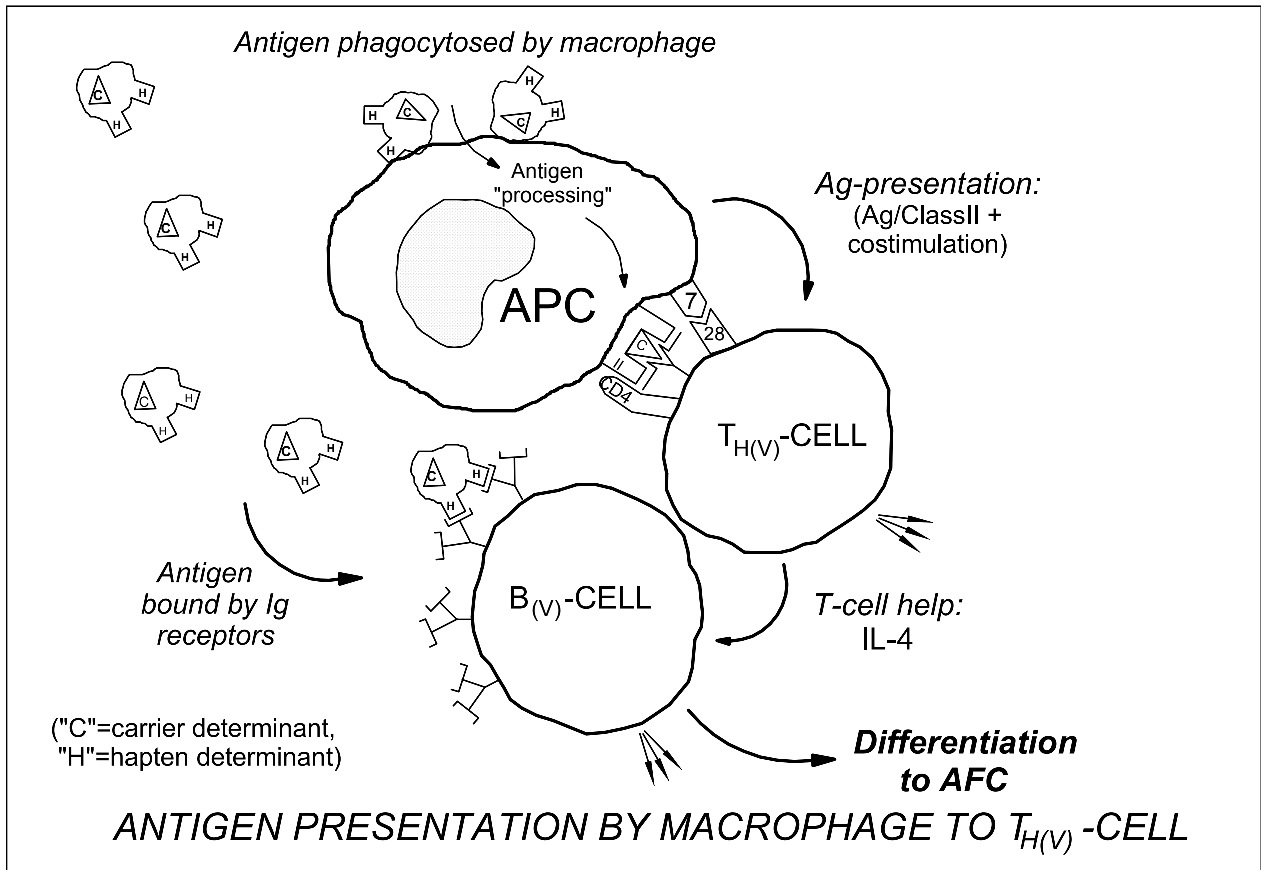
A *primary* humoral immune response requires the cooperation of three cell types, an ANTIGEN-PRESENTING CELL (APC, typically a dendritic cell or macrophage), a T-HELPER CELL (TH2), and a B-CELL. The APC/T-CELL interaction involves the recognition of MHC Class II-bound peptide by the T-cell receptor, and the participation of the accessory molecule CD4. The same principles apply to *secondary* humoral responses, except that the B-cell can also take on the role of APC, presenting processed antigen to memory T-cells; thus, only two cells (B-cell and memory T-cell) need participate in this interaction. This two-cell interaction is the basis for understanding the experimental phenomenon of the CARRIER EFFECT.

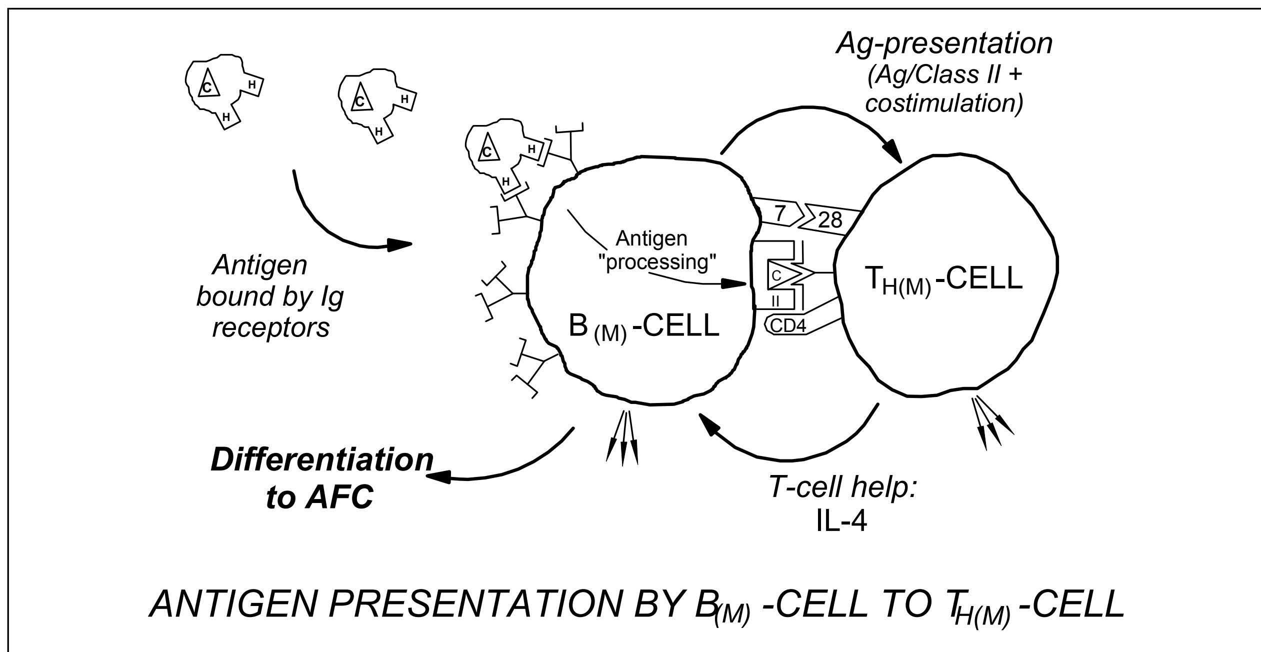
"THREE-CELL INTERACTION" IN IMMUNE RESPONSES

The generation of immune responses generally requires the participation of *three classes of lymphoid cells*, an *antigen-presenting cell* (APC), a *helper cell* and an *effector cell*. We have seen one example of this in the generation of cell-mediated immunity outlined in Chapter 12; in that case the APC was a macrophage or dendritic cell, the helper was a T_{H1} cell, and the effector was a T_C cell.

The same principle holds in the cell interactions required for the development of humoral responses. At the top of Figure 15-1 is shown such an interaction, involving an APC, a T_{H2} cell (a *virgin* T_{H2} cell, as opposed to a memory cell, discussed further below), and a B-cell. This interaction is similar in most respects to the one we have already seen in Chapter 12. The APC bears *surface antigen associated with Class II MHC molecules* which is recognized by the T_H cell; the T-cell receptor provides the antigen-specific recognition, and the *CD4/Class II contact* stabilizes this interaction. The antigen-presenting cell provides a "costimulatory" signal to the T-cell in the form of its membrane B7 molecule (which is recognized and bound by CD28 on the T_H-cell) and the T_H cell then responds by *secreting IL-4*. The cell which responds to this IL-4 is a nearby B-cell, which bears membrane Ig receptors (specific for LCMV) which have already been occupied and cross-linked by antigen; this *B-cell responds to IL-4 by differentiating into an antibody-forming cell* and undergoing clonal expansion.

Memory T_H-cells ($T_{H(M)}$-cells) can participate in a second kind of interaction, illustrated at the bottom of Figure 15-1. This two-cell interaction results from the fact that **B-cells are capable of acting as efficient APC's for memory T_H cells**. (It's worth noting that we might have predicted this, based on the fact that B-cells constitutively bear Class II molecules on their surface.) In this interaction, the *B-cell plays two roles*. First, it acts as an *APC*, by presenting Class-II-associated viral antigen to the $T_{H(M)}$-cell and expressing the costimulatory molecule B7. Second, it then acts as an *effector cell* by responding to the IL-4 secreted by the T-cell, and it undergoes differentiation and clonal expansion. Thus, this interaction can still be conceptually regarded as a "three-cell" interaction (APC/Helper/Effector), despite the fact that only two distinct cells participate.





CELL INTERACTIONS IN HUMORAL IMMUNITY

Figure 15-1

T/B-CELL COOPERATION

The special importance of B-cells as APCs for memory T-cells stems from the fact that they are clonally precommitted; those rare memory B-cells (perhaps one in a thousand) which happen to be specific for an epitope on a particular antigen *can very effectively bind that antigen* by their membrane Ig receptors. Having bound the antigen, the B-cell then internalizes it (by *receptor-mediated endocytosis*, not phagocytosis), associates its degraded peptides with newly synthesized Class II MHC molecules, and displays these new class II molecules on its surface. Those memory T_H cells which happen to be clonally precommitted to *carrier* epitopes on the antigen (also rare cells) can recognize the Class II-associated carrier determinants via their T-cell receptors. (Why such B-cell-dependent antigen presentation plays only a small role in primary responses is not immediately obvious, since B-cells' advantages as APCs should in principle still hold; it may be that prior to clonal expansion and affinity maturation they are too rare or bind antigen too poorly to play a more effective role.)

The signaling which then follows is the same as we have already seen in CMI, namely the transmission of a co-stimulatory signal by the APC (B-cell), which triggers the helper T cell to produce IL-4, which then in turn stimulates the B-cell (which bears IL-4 receptors). Thus, **two signals** are necessary for triggering a B-cell to differentiate into an effector (=Ab-secreting) cell: first, its *antigen-specific Ig receptors must be occupied and cross-linked*; second, it must receive *appropriate signals* (*e.g*. IL-4) from a helper cell. As we have seen, the requirement for two signals is analogous to that of both T_{H1} and T_C cells in CMI.

For antibody production to T-dependent antigens, *T-cell/B-cell interaction is required for both primary and secondary responses* although the nature of the interactions appears to differ in the two cases (as we have seen in Figure 15-1). We will illustrate some of the consequences of this model as applied to **secondary humoral responses** by two experiments which, more than 40 years ago, defined the CARRIER EFFECT.

EXPERIMENTAL ANALYSIS OF THE CARRIER EFFECT

The key features of the T-cell/B-cell interaction for humoral immune responses, which we will illustrate using the Carrier Effect, are as follows:

1) *Two classes of lymphocyte are required, TH-cells and B-cells*.

2) *T and B-cells recognize different epitopes, or "antigenic determinants", on the same antigen molecule*. Those recognized by B-cells we call **hapten** determinants, those recognized by T-cells are **carrier** determinants.

We can illustrate these requirements with two simple experiments in mice, using the DNP (dinitrophenyl) group as a hapten, and three unrelated (*i.e*. non-cross-reactive) proteins as carriers, namely bovine gamma globulin [BGG], bovine serum albumin [BSA] and chicken ovalbumin [OVA]

In order to understand these experiments, it's important to keep in mind two basic features.

1. **We are measuring only antibody (humoral) responses to the hapten DNP**. Humoral immune responses against the carrier will also take place, but we will ignore them. Cell-mediated responses against the carrier may also be generated, but we will not concern ourselves with these either.

2. **We are measuring only secondary, memory responses**, which result in *high levels of IgG antibody*. Primary humoral responses to DNP will certainly be generated, but these will produce only low levels of IgM and, again, we will ignore them.

As will be illustrated in the experiments below, **generation of these secondary responses requires the presence of both memory B-cells and memory T-cells**, each of which must have been generated by a previous primary immunization.

EXPERIMENT 1: CARRIER EFFECT

The host must be primed to both hapten and carrier to generate a response, and must be challenged with both on the same molecule.

We immunize ("prime") mice with various antigens, then challenge ("boost") two weeks later. We then test their serum for the presence of anti-DNP antibodies one week after the boost.

	Prime	Challenge	Test for *anti-DNP* Ab
1)	DNP alone	DNP-BGG	-
2)	DNP-BGG	DNP-BGG	+
3)	DNP-BGG	DNP-BSA	-
4)	DNP-BGG	BGG	-
5)	DNP-BGG	DNP-BSA + BGG	-

As can be seen in line 3, priming with DNP-BGG will *not* result in an anti-DNP response to a boost with DNP on *another* carrier (BSA). This defines the **carrier effect**; the host's immune status toward the carrier affects the response to the hapten.

EXPERIMENT 2: SEPARATE CARRIER AND HAPTEN PRIMING

The host must have been primed to *both the carrier and the hapten* used for challenge; however, the *priming can be carried out separately for the two kinds of epitopes*.

We will give the mice *two* primary immunizations; these can be given simultaneously (in two different sites) or a week apart. We will then continue as before, challenging after two weeks then testing for the presence of anti-DNP antibody one week later.

	Primary Immunizations #1	#2	Challenge	Anti-DNP Antibody
1)	DNP-OVA	–	DNP-OVA	+
2)	DNP-OVA	–	DNP-BGG	–
3)	DNP-OVA	BGG	DNP-BGG	+
4)	DNP-BGG	OVA	OVA	–
5)	DNP-BGG	OVA	DNP-OVA	+
	etc.			

In order to produce a secondary anti-DNP response after challenge with DNP-BGG, the animal must have been primed to *both* DNP and BGG (line 2); however, as seen in line 3, the animal can be *separately* immunized to DNP (on *any* carrier) and BGG (without DNP). Therefore, the *recognition of hapten and carrier determinants must be executed by separate cell populations*, since they can be separately primed.

By carrying out additional experiments not shown here, one can demonstrate that those primed *cells required for recognition of the carrier are T-cells (i.e.* they can be killed with antibodies to the mouse T-cell-specific antigen Thy-1), while *those cells recognizing the hapten are B-cells* (not affected by anti Thy-1 treatment).

The basic feature of T-cell B-cell cooperation that is demonstrated here is that *two different cells need to recognize different antigenic specificities on the immunogen* in order to cooperate. The names "hapten" and "carrier" in this context refer simply to those epitopes recognized by B-cells and T-cells, respectively, but some clarification may be useful. The epitope recognized by the B-cells does not necessarily have to be a “hapten” in the strict sense of the term (*i.e.* a small chemical moiety which can be coupled to different carriers). Any suitable target, including portions of a “carrier” protein molecule, could be recognized by B-cells and serve as the “hapten” portion of the hapten/carrier phenomenon; the use of a “moveable” classic hapten such as DNP simply makes the design and interpretation of such experiments much easier.

The “carrier” specificity, however, has more limited possibilities. In theory, T-cells could use DNP as a "carrier" specificity and could then cooperate with B-cells for an antibody response to some other (or the same) "hapten" determinant present on the BSA molecule. However, DNP and other typical haptens *cannot* be recognized by T-cells, because they are not peptides and therefore cannot bind to the peptide-binding groove of an MHC molecule (see Chapter 12). Thus, while B-cells using their cell surface immunoglobulin can bind to *both* peptide antigens, either free or within a larger protein, and haptens such as DNP, T-cells, which use their TCR for antigen recognition, are largely restricted to recognizing only *peptides* intimately associated with a suitable MHC molecule.

TWO KINDS OF T-INDEPENDENT ANTIGENS: *TI-1* AND *TI-2*

The cell interactions shown in Figure 15-1 and illustrated by the Carrier Effect are required for humoral responses to *T-dependent (TD)* antigens (see Chapter 13). As already mentioned, however, there are some antigens which do not require T-cell help to generate a humoral

immune response, and these are known as *T-independent (TI)* antigens. Two general classes of T-independent antigens can be distinguished, which have been named *TI-1* and *TI-2*.

TI-1 antigens are exemplified by *lipopolysaccharide* (LPS), a component of the cell wall of Gram-negative bacteria. LPS is mitogenic for B-cells, and can therefore efficiently activate *any* B-cell with LPS-binding antigen receptors, even if the LPS is present only at very low concentrations, which results in an antibody response. In effect, the mitogenic activity of LPS replaces the need for the “second signal” normally provided by the T_H cell.

TI-2 antigens, on the other hand, are not mitogenic, but typically bear highly repetitive epitopes (for example, high molecular weight carbohydrates). The exceptionally high degree of Ig-receptor cross-linking produced by such antigens seems to provide a very high level of “Signal 1”, and reduces or eliminates altogether the requirement for “Signal 2” (normally provided by IL-4 and other lymphokines). However, in the absence of antigen-specific T-cell help, humoral immune responses to TI antigens of either type generally do not exhibit class switching, and are therefore largely restricted to triggering IgM responses (as, for example, the ABO blood group antigens discussed in Chapter 10).

CHAPTER 15, STUDY QUESTIONS:

1. Describe the different cell/cell interactions involved in PRIMARY versus SECONDARY humoral responses.
2. What are the critical cellular requirements for generating an IgG ANTI-HAPTEN HUMORAL RESPONSE in response to the injection of DNP coupled to a carrier?
3. Define and distinguish T_{H1} and T_{H2} cells.
4. Define and describe the two classes of *T-independent* antigens.

CHAPTER 16
LYMPHOID TISSUE STRUCTURE

Beginning with mouse lymph node as the prototype, we examine some fundamental features of all secondary LYMPHOID TISSUES, particularly the segregation of T-DEPENDENT REGIONS containing T-cells (DIFFUSE CORTEX) from T-INDEPENDENT REGIONS containing B-cells (PRIMARY FOLLICLES). The process of MIGRATION (the exit of mature B-cells from the bone marrow, the movement of T-cell precursors from bone-marrow to thymus, and the emigration of mature T-cells from the thymus) is distinguished from that of RECIRCULATION (the process by which both B- and T-cells continually enter lymphoid tissues from the blood and return to the blood via the lymph). The formation of GERMINAL CENTERS in pre-existing primary follicles is seen to be an antigen-dependent and T-cell-dependent process.

Other peripheral lymphoid tissues (*e.g.* spleen, Peyer's patches), and lymphoid tissues of other species including humans, although differing in details, all show the same basic features of structural organization and cell movement.

Immune responses are not carried out in any single organ, but in a wide variety of structures collectively known as LYMPHOID TISSUE. Lymphoid tissue can be generally categorized as CENTRAL (or ”PRIMARY”), versus PERIPHERAL (or ”SECONDARY”). Central lymphoid tissues are those which act as a *source of immunocompetent cells*; these cells then migrate to the peripheral lymphoid tissues which are the *sites of immune responses*.

Central	***Peripheral***
Thymus	*Lymph Node*
Bone Marrow	*Spleen*
	Gut-Associated lymphoid tissue (GALT)
	Tonsils
	Appendix
	Peyer's Patches

The lymphoid tissues we will be discussing are broadly included in the term **Reticuloendothelial System**, or **RES**. The RES also includes other regions rich in phagocytic cells, notably those in the vasculature of the liver and lungs.

MOUSE LYMPH NODE

We will examine the structure of a typical mouse lymph node (diagrammed on next page) to illustrate several key points: Lymphoid tissue is not just a "bag" of lymphoid cells, but consists of a highly ordered structure; it is also a *dynamic* structure, maintained by a continuous movement of cells into and out of the tissue (via MIGRATION and RECIRCULATION); characteristic anatomical changes can be observed during the generation of an immune response.

The structure of a mouse lymph node can be divided into two areas, CORTEX and MEDULLA.

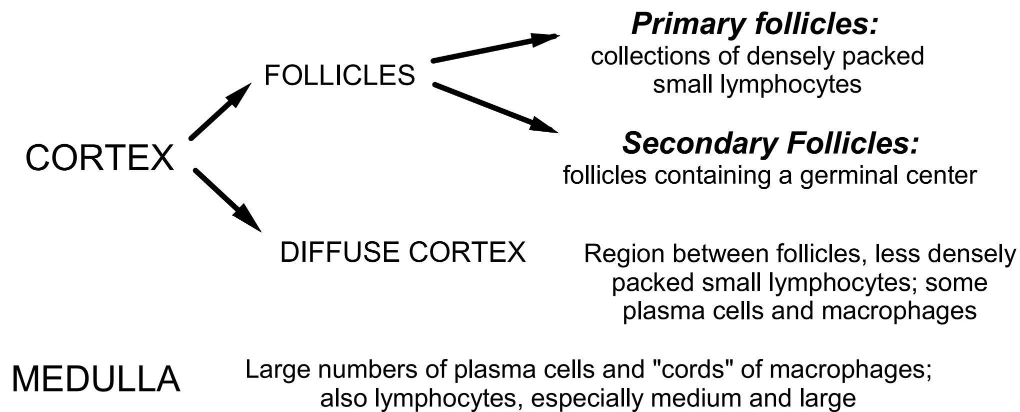
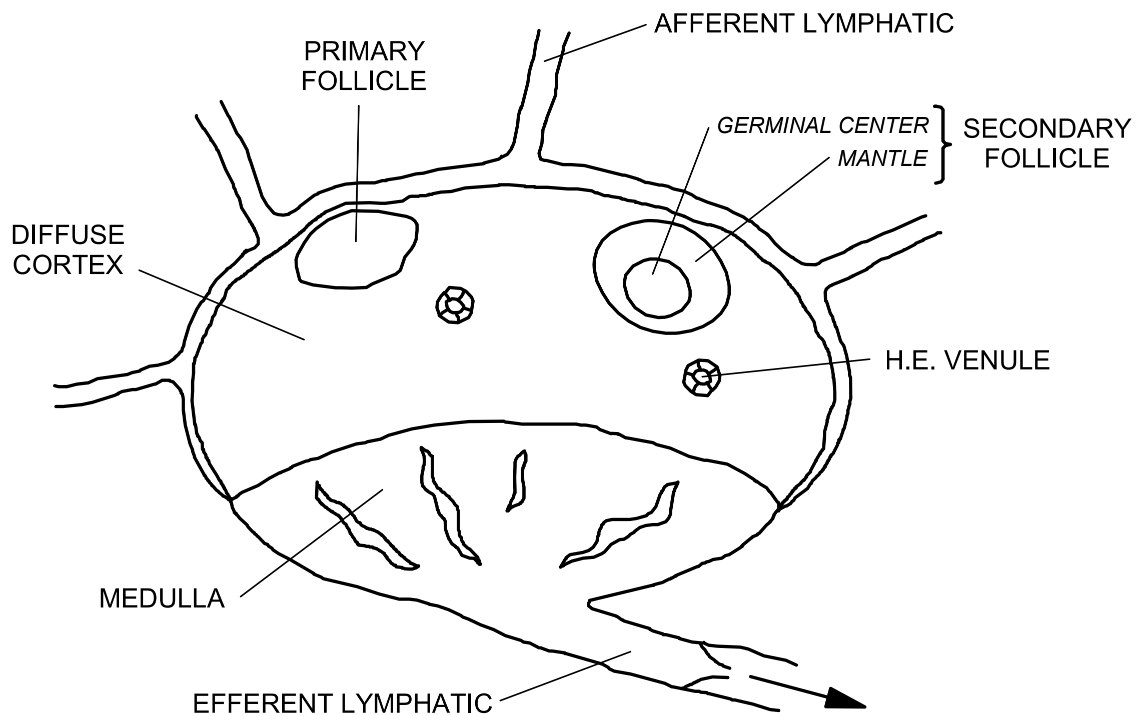


Figure 16-1



ARCHITECTURE OF A MOUSE LYMPH NODE

Figure 16-2

As fluids from various tissues are collected in the lymphatics, they enter the afferent vessels of a lymph node, percolate through the cortex and through the medulla, and are collected in the single efferent lymphatic through which they leave the node; this fluid eventually ends up in one of the major lymphatics (*e.g*., thoracic duct) and is dumped into the venous circulation.

The Primary Follicles of the cortex are made up primarily of B-lymphocytes (with occasional T-cells) and are characteristic of a resting or unstimulated node. Upon *antigenic stimulation*, normally following entry of antigen via the afferent lymphatic fluid, a region of intense proliferation develops within the follicle, known as a Germinal Center; the germinal center displaces the remaining densely packed B-cells into a peripheral "mantle", and the follicle is now known as a Secondary Follicle. In addition to containing proliferating cells, the *germinal center* also is a site of *cell death*, and one diagnostic feature is the presence of macrophages which contain phagocytosed cell debris *("Tingible Body Macrophages"*).

The Diffuse Cortex contains mainly T-lymphocytes. In neonatally thymectomized mice (also in *nu/nu* athymic mice and congenitally athymic humans) this area is virtually empty of lymphocytes, and for this reason became known as a THYMUS-DEPENDENT AREA (TDA) of lymphoid tissue. In young mice (and continuously, although to a lesser extent in adult animals) mature T-cells which develop within the thymus leave that organ, enter the circulation and colonize the T-dependent areas of lymph nodes and other peripheral lymphoid tissues; this process is one manifestation of lymphocyte MIGRATION.

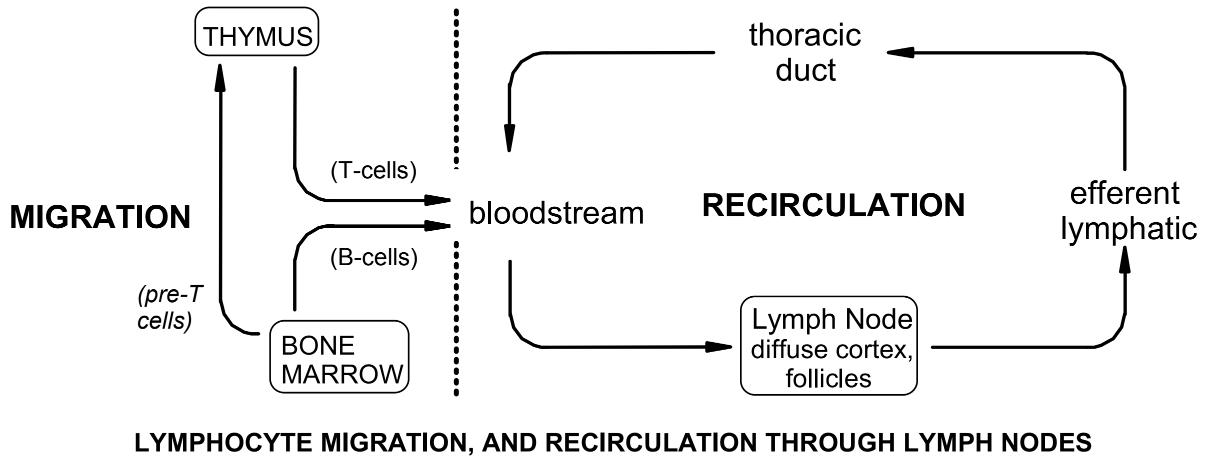
A second consequence of neonatal thymectomy is the lack of development of germinal centers upon antigenic stimulation (and, of course, a lack of immune responsiveness to T-dependent antigens). *Thus, although germinal centers develop within B-cell areas (primary follicles), they are T-dependent in their development*.

LYMPHOCYTE RECIRCULATION

Lymphocytes leave the lymph node continuously through the efferent lymphatic, enter the blood circulation, and re-enter the lymph nodes through specialized vessels known as High Endothelial Venules (HEVs); this process is known as RECIRCULATION. If the thoracic duct (a major lymphatic vessel) of a mouse is cannulated and the lymph (and the cells within it) is removed for a period of a week, the diffuse cortex becomes emptied, just as if the animal had been neonatally thymectomized. This is because T-cells recirculate more rapidly than B-cells and are therefore more readily depleted; a longer period of cannulation will eventually remove the B-cells as well.

The entry of lymphocytes into lymph nodes is a highly specific process, involving recognition by lymphocytes of receptor molecules on the endothelial cells of the HEVs; erythrocytes, granulocytes and other cells are not capable of carrying out this process. As their name implies, the HEVs are characterized by high cuboidal endothelial cells instead of the squamous cells commonly lining other vessels, and they are present in some other peripheral lymphoid tissues (*e.g*. Peyer's patches) as well. Recirculation of lymphocytes, in fact, is a universal characteristic of peripheral lymphoid tissues. (NOTE: Recirculation of lymphocytes also occurs through the spleen, although this organ does not contain HEVs. In this case lymphocytes enter the spleen by a morphologically distinct route in the "marginal sinus" which surrounds the follicles.)

A diagrammatic representation of lymphocyte migration and recirculation through lymph nodes is shown below. Keep in mind that these processes occur in *all* peripheral lymphoid tissues (spleen, Peyer's patches, *etc.*), and that all of them contain follicles as well as thymus-dependent areas.



LYMPHOCYTE MIGRATION, AND RECIRCULATION THROUGH LYMPH NODES

Figure 16-3

One additional migration pathway which should be mentioned is the one by which *bone marrow-derived pre-T cells enter the thymus* (this is also shown in the diagram). These are the cells which following entry into the thymus differentiate into mature T-cells, undergoing positive and negative selection to ensure that only suitable T-cell receptors are expressed, and eventually migrate back out into the periphery. This process occurs at a high rate during the early development of the immune system, but persists at a lower rate throughout the life of the animal despite the overall involution of the thymus. (See Chapter 18, TOLERANCE for a more detailed discussion.)

TIME COURSE OF EVENTS IN A "TYPICAL" IMMUNE RESPONSE

Day 0 Antigen introduced in a local site (*e.g.*, skin of the foot).

Day 1 Antigen found within the draining lymph node (*e.g.*, popliteal), having been carried in by activated dendritic cells in the tissues, as well as taken up by resident dendritic cells in the diffuse cortex (also by phagocytic cells of the medulla). These cortical dendritic cells are highly efficient antigen-presenting cells, and it is here that the initial interaction involving antigen-specific T_H and B-cells is thought to occur.

Day 1-2 Lymph node enlarges, due to increased entry of cells from circulation and decreased exit via lymphatics (*i.e.* inflammatory response).

Day 2 Wave of T-cell mitosis followed by B-cell mitosis.

Day 4 Antibody-forming cells begin to appear in diffuse cortex.

Day 4-5 Early germinal center formation, site of class-switching and somatic mutation.

Day 5-6 Early antibody-forming cells exit lymph node via efferent lymphatic, colonize distant lymphoid tissues (spleen, other lymph nodes); large numbers of antibody-producing plasma cells begin to appear in medulla.

The following points should be noted:

- The mitoses seen in the local node represent *clonal expansion* of antigen-specific precommitted T-cells and B-cells, as well as non-specific proliferation resulting from the many lymphokines present.
- The rapid recirculation of lymphocytes (particularly of T-cells) allows the *recruitment* of antigen-specific cells to the local site. The enlargement of the node represents the result of such recruitment, in addition to a great deal of non-specific accumulation of cells and fluid.
- Although the initial response is generated within the lymph nodes which drain the local site of antigen deposition, the antibody-forming cells which are produced rapidly spread to other sites, and memory cells rapidly enter the recirculating pool as well as colonize other peripheral lymphoid tissues; the net result is effective *systemic immune responsiveness* and memory.
- Germinal Centers are a major site for the generation of memory B-cells, isotype switching and somatic generation of diversity for immunoglobulins. While "follicles" are defined as regions of B-cell localization, it is important to remember that *germinal centers are both T-dependent and antigen-dependent in their formation*.
- As has already been discussed, there exists a class of antigens ("*T-independent antigens*") which do not depend on T-cell function to generate an antibody response. The response to such antigens does not lead to germinal center formation, and the histological events in such responses will not be discussed here.

CHAPTER 16, STUDY QUESTIONS:

1. Follow a lymphocyte from the bloodstream through its RECIRCULATION pathway(s) back to the blood; identify where it goes and what specific cell interactions must take place during this process. How do T and B-cells differ in this regard?
2. What cellular and molecular events take place in the GERMINAL CENTER? Do all immune responses result in GERMINAL CENTER FORMATION?

CHAPTER 17
ONTOGENY OF THE IMMUNE SYSTEM

HEMATOPOIETIC STEM CELLS originate in the yolk sac of the developing embryo, migrate early into the FETAL LIVER, and later into the BONE MARROW, which is the only normal site of hematopoiesis in the adult. The generation of immunocompetent cells from hematopoietic precursors in the bone marrow is a process which continues throughout the life of an individual. Human infants are born with a functioning immune system, and are additionally protected by transplacentally acquired maternal IgG for the first few months of life. Exchange of cells and immunoglobulins between mother and fetus takes place during gestation, which may result in MATERNAL IMMUNIZATION to HLA and blood group antigens, and the pathological consequences of Rh INCOMPATIBILITY (discussed in Chapter 10).

A discussion of the "development" of an organ system would normally consist simply of a description of its embryological origins. However, as we have already noted, the entire hematopoietic system (including the immune system) is in a *continuous* state of regeneration throughout life, and is therefore continuously being "developed." It is largely in this context that we will discuss the ontogeny of the immune system. We will also discuss some important aspects of the immunological relationships between mother and fetus; keep in mind, however, that this relationship is still only poorly understood, and is much more complex than is being discussed here.

ORIGIN OF HEMATOPOIETIC STEM CELLS

All cells of the hematopoietic system are continuously being generated from a single kind of precursor cell known as the **Hematopoietic Stem Cell.** This stem cell is capable of unlimited mitotic cell division, more specifically *asymmetric* division which results in two classes of products. One class includes cells in various stages of differentiation, eventually yielding each of the mature cells of the blood and immune system including lymphocytes (both B- and T-cells), granulocytes, monocytes, red blood cells and platelets. A second class of product is represented by new stem cells identical to the parent cells. The stem cell is therefore said to exhibit the property of **self-renewal**; in fact, self-renewal is a *defining* property of stem cells. This continuous regeneration of immunologically competent cells has many important consequences, not the least of which is the fact that whatever processes are required to maintain the normal state of TOLERANCE (see Chapter 18) must take place not only during embryological development, but *continuously throughout life*.

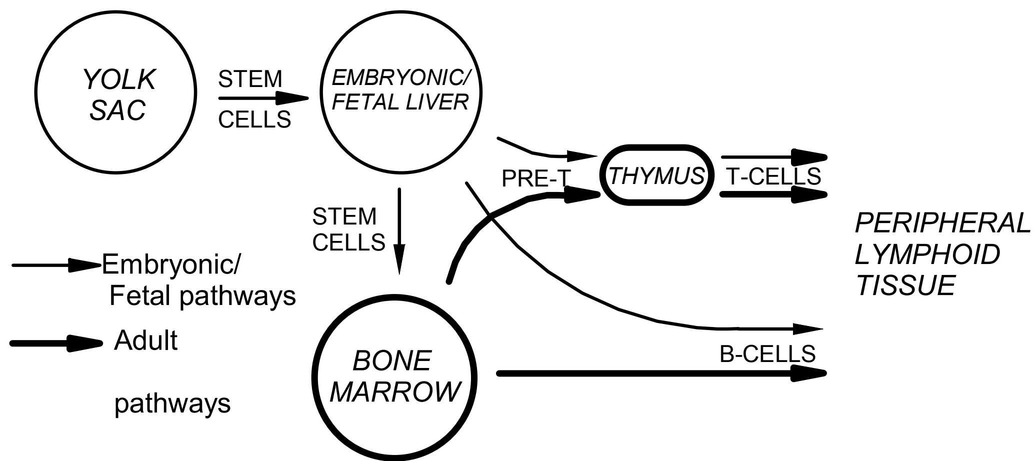


Figure 17-1

In normal human adults, the generation of *all* cells of the hematopoietic system, with one important exception, is restricted to the bone marrow. We've already discussed this exception in Chapter 13; while B-cells (and most other blood cells) are produced within the bone marrow, mature T-cells are produced exclusively *within the thymus*, from precursors ("pre-T-cells") which themselves are bone-marrow-derived and have entered the thymus from the blood.

The question of the origin of the immune system therefore can, in one sense, be reduced to the question of the origin of stem cells. As shown in Figure 17-1, the first stem cells (and the first blood cells) appear early in the course of human embryological development (at about two weeks of gestation) in the blood islands of the **yolk sac**. As the developing blood vessels begin making connections with the embryo itself, stem cells move into the developing **fetal liver**, the first hematopoietic organ of the embryo, and transiently into the spleen. By the time of birth, neither the liver nor the spleen remains a site of hematopoiesis in humans; the stem cells have migrated into the **bone marrow**, which remains the normal site of generation of all blood cells throughout life. This *movement of stem cells from the yolk sac to the embryonic liver, and then to the bone marrow*, adds two new paths to the patterns of cell **migration** we have already discussed in Chapter 16.

[NOTE: In certain pathological conditions, some anemias for example, development of blood cells may take place at sites other than the bone marrow, a condition referred to as *"extramedullary hematopoiesis"*. In other species (mice, for example) the normal spleen may retain its hematopoietic role throughout life; it is for this reason that mouse spleen contains not only B- and T-cells characteristic of normal peripheral lymphoid tissue, but also hematopoietic stem cells capable of rescuing a lethally irradiated animal from hematopoietic death.]

IMMUNOLOGICAL STATUS OF THE NEWBORN

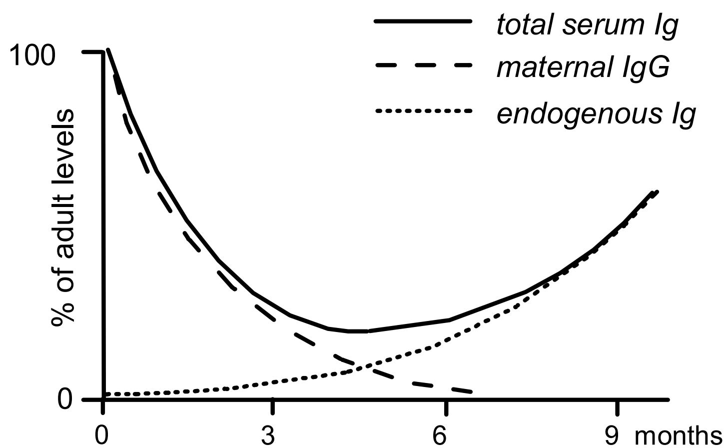
Fetal Development of Immunity

Mice are born at an immunologically very immature stage of development; few B-cells are found in the peripheral lymphoid tissue, and almost no T-cells. This explains the fact that removal of the thymus immediately after birth results in a profound and permanent T-cell deficiency in mice (as we discussed in Chapter 13), and accounts for the sensitivity of newborn mice to developing GvH.

Humans, on the other hand, are born with considerably more mature immune systems; the newborn human is capable of generating effective immune responses, both humoral and cellular, although not necessarily at adult levels. T-cell and B-cell areas of peripheral lymphoid tissue are already largely populated, and show the same basic architecture seen in adult tissues. (NOTE: One characteristic feature of newborn human peripheral lymphoid tissue is the presence of *primary* follicles. These are rare or absent later in life, due to the normal antigenic stimulation of these tissues, and the consequent development of germinal centers and *secondary* follicles).

Maternal IgG in the Newborn

If one examines the level and nature of immunoglobulins in the serum of a newborn human, an interesting situation becomes apparent. While the total Ig in newborn serum is at a level close to that of a normal adult, almost all of it is *IgG of maternal origin*. This results from the fact that IgG (but not other classes) can be transported across the placenta, passing from the maternal circulation to that of the fetus via a transport mechanism involving specific Fc-receptors in the placenta. This process begins around the 22nd week of gestation and continues to term.



SERUM IMMUNOGLOBULIN IN THE NEWBORN

Figure 17-2

As shown in the figure above, the only immunoglobulin normally present at substantial levels in the newborn is maternal IgG (this can be determined by examination of allotypes). Small amounts of IgM and trace amounts of IgA are also present; since these immunoglobulins do

not cross the placental barrier, they must be of fetal origin; their presence in the newborn circulation at high levels (detected by its presence in umbilical cord blood) is considered a sign of *intrauterine infection* and a resulting fetal immune response.

Mature B-cells and T-cells are already present at the time of birth, but it is only after birth and exposure to environmental antigens that they normally begin to generate appreciable immune responses. As a result, the levels of *endogenous* (as opposed to maternal) serum immunoglobulin begin to rise during the first few months after birth, IgM rising earliest, followed by IgG and IgA.

While endogenous synthesis of immunoglobulins is already underway at birth, it takes several months to reach levels which can effectively replace the protection conferred by the passively acquired maternal IgG. This maternal IgG disappears with a normal half-life of 2-3 weeks, and (of course) is not replenished. A low point in total serum Ig is typically reached at about 4-5 months of age, which is also the time at which humoral immunodeficiencies may become clinically evident (see Bruton's Agammaglobulinemia in Chapter 20).

> NOTE: The newborn infant is also protected by maternal IgA which it acquires from its mother's milk (particularly the early form known as *colostrum*). However, while this IgA plays an important role by protecting against infection by gut-localized pathogens, *this IgA does not enter the infant's circulation*.

Maternal/Fetal Interactions:

The developing fetus can be regarded as a graft of "foreign" tissue onto the mother; it is clearly a **histoincompatible graft**, since at least some HLA antigens (those of paternal origin) will be foreign to the mother. If this is so, why is the fetus not recognized as foreign and rejected? In fact, the fetus *is* generally recognized as foreign by the mother's immune system, but is nevertheless not rejected. There are several (and still poorly understood) reasons for this.

First, the placenta itself may act as a **filter** for anti-HLA antibodies; maternal antibodies directed against paternal HLA antigens present in the fetal component of the placenta may be bound by the placental tissue. The placenta is not harmed by these antibodies, but it prevents their passage into the fetal circulation where they might be harmful, thus effectively neutralizing the mother's *humoral immune response* to the fetus. *Second*, this "coating" of antibody on paternal antigens present in the placenta may "hide" the foreign HLA antigens and prevent recognition and damage by the mother's *cell-mediated immune response* (in the manner of *enhancing antibodies*; see Chapters 11 and 23). *Third*, cells of the outermost layer of the placenta, the trophoblast, do not express the HLA Class I proteins which are present on all other nucleated cells, thus reducing the generation of, and the target for, anti-HLA cell-mediated responses by the mother. And *fourth*, the state of pregnancy induces a state of moderate **immunosuppression** of the mother (by various mechanisms), which has the effect of further discouraging anti-fetal responses.

While the placenta does a relatively good job of keeping the maternal and fetal sides of the circulation separate, several materials of immunological importance *can* cross the placental barrier to various extents, as outlined in Figure 17-3, below.

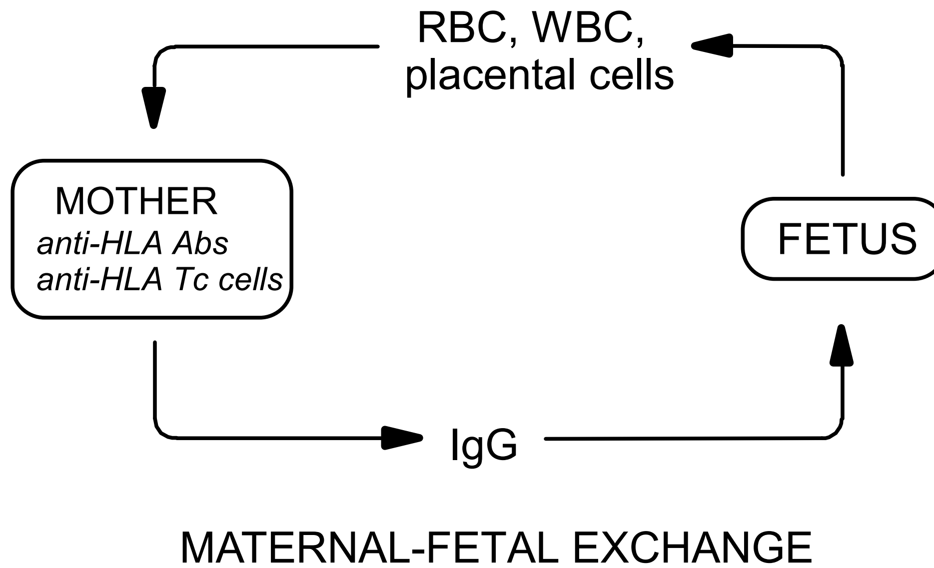


Figure 17-3

One example, as we have already seen, is that of *maternal IgG*, which is efficiently transported into the fetal circulation before birth; this IgG is critically important for protection of the newborn during its first few months of life. Another example is that of small numbers of *cells of fetal origin* (red blood cells as well as nucleated cells) which enter the maternal circulation, presumably through microscopic defects in the placental barrier. These cells are of interest for at least two reasons. *First*, they may eventually stimulate substantial anti-HLA antibody responses in the mother (already mentioned above), particularly after multiple pregnancies; in fact, *multiparous women* are an important source of the anti-HLA antibodies which are used in histocompatibility typing. *Second*, these rare cells can be identified and isolated from the mother's circulation; their use for prenatal testing of genetic disease, which would eliminate the need for the more expensive and hazardous process of amniocentesis, is currently under development. (Red blood cells may also trigger maternal antibody responses directed against blood group antigens, although such a response is far stronger after the appearance of the larger number of red blood cells which enter the maternal circulation at the time of birth and the separation of the placenta. The development and significance of maternal anti-Rh responses have been discussed in Chapter 10).

CHAPTER 17, STUDY QUESTIONS:

1. Follow a MEMORY T- or B-CELL back in time through its development; what are the membrane markers and anatomical localizations associated with each of its identifiable precursors?
2. How would you use heavy and light chain Ig ALLOTYPES to distinguish the origins of serum Ig in a *newborn*? How would your results be different if you looked at a 6-month-old or 2-year-old child?
3. Why are multiparous women not a good source for ANTI-Gm ANTIBODIES

CHAPTER 18
TOLERANCE

TOLERANCE (which is *antigen-specific*) must be distinguished from immune deficiency (non-specific). Its most important manifestation, the maintenance of SELF-TOLERANCE, was originally explained by the Clonal Selection theory as the result of CLONAL ABORTION of self-reactive clones. However, potentially self-reactive T- and B-cells *do* exist in normal individuals, leading to the recognition of the importance of active processes in the maintenance of tolerance, such as SUPPRESSION. The dynamic and competing *balance between immunity and tolerance* is discussed, together with three useful model systems, INDUCED CLONAL ABORTION, RECEPTOR BLOCKADE, and ANTIGEN SEQUESTRATION. Two general categories of tolerance are recognized, CENTRAL TOLERANCE which is generated during development of T-cells within the thymus, and PERIPHERAL TOLERANCE generated by a variety of mechanisms outside this central lymphoid organ.

One of the defining features of the immune system is its ability to *distinguish self from non-self*--that is, it must be capable of mounting a reaction against any *foreign* antigen, but *not* respond to substances normally present in the organism itself. This essentially describes the phenomenon of **natural tolerance**.

TOLERANCE – Lack of ability of an organism to mount an immune response *to a specific antigen*.

Antigen-specificity is a key part of the definition of tolerance, and distinguishes it from the phenomena of **immunosuppression** or **immunodeficiency** in which immune reactivity is diminished to *all* antigens.

EARLIEST ANALYSIS OF TOLERANCE: DIZYGOTIC CATTLE TWINS

The beginning of our modern understanding of tolerance dates back to the experiments of Ray Owen in the 1940's. He was studying the genetics of blood groups in cattle, and found that *dizygotic* (*i.e*. non-identical) *twins* were often *chimeric*, showing *mixtures* of genetically distinct blood groups in their circulation. This was because such cattle twins often shared a single placenta which resulted in a mixing of embryonic blood between them. Each had erythrocytes of its own blood groups, plus a varying number of cells of its twin's blood groups which persisted throughout life. (NOTE: This is very different from most human dizygotic twins which *do not* share a common placenta.)

Owen noted a remarkable feature of this chimerism, namely that *neither twin made antibodies against the blood cells of the other*, which they would have done had they been exposed only as adults to the foreign blood cells. In addition, he was able to show that *neither twin would reject a tissue graft from the other* as adults, which they also would certainly do under normal circumstances. He hypothesized that *exposure of the immature immune system to a foreign antigen resulted in specific tolerance to that antigen*. This concept was soon confirmed by Medawar's experiments in mice, and was a central part of Burnet's Clonal Selection theory published in 1959.

As we have mentioned earlier, Burnet in 1959 proposed the mechanism of CLONAL ABORTION as an explanation of *normal self-tolerance*. This simply represents the elimination, at an early stage of development, of all cells specifically precommitted to any antigen which is present at that stage. In the case of B-cells (remember that the existence of T-cells was not known at the time) this concept is fairly straightforward, and clonal abortion is now accepted as being an important mechanism for establishing and maintaining B-cell tolerance. However, it is more difficult to understand how clonal abortion might operate in the case of T-cells, given the necessity of their recognizing antigen *strictly* in an MHC-associated fashion (as we discussed in Chapters 14 and 15).

POSITIVE AND NEGATIVE SELECTION OF T-CELLS WITHIN THE THYMUS

A key to understanding the normal functioning of T-cells, as well as their self-tolerance, is the process of positive and negative selection which they undergo during their maturation within the thymus, as shown in Figure 19-1, below.

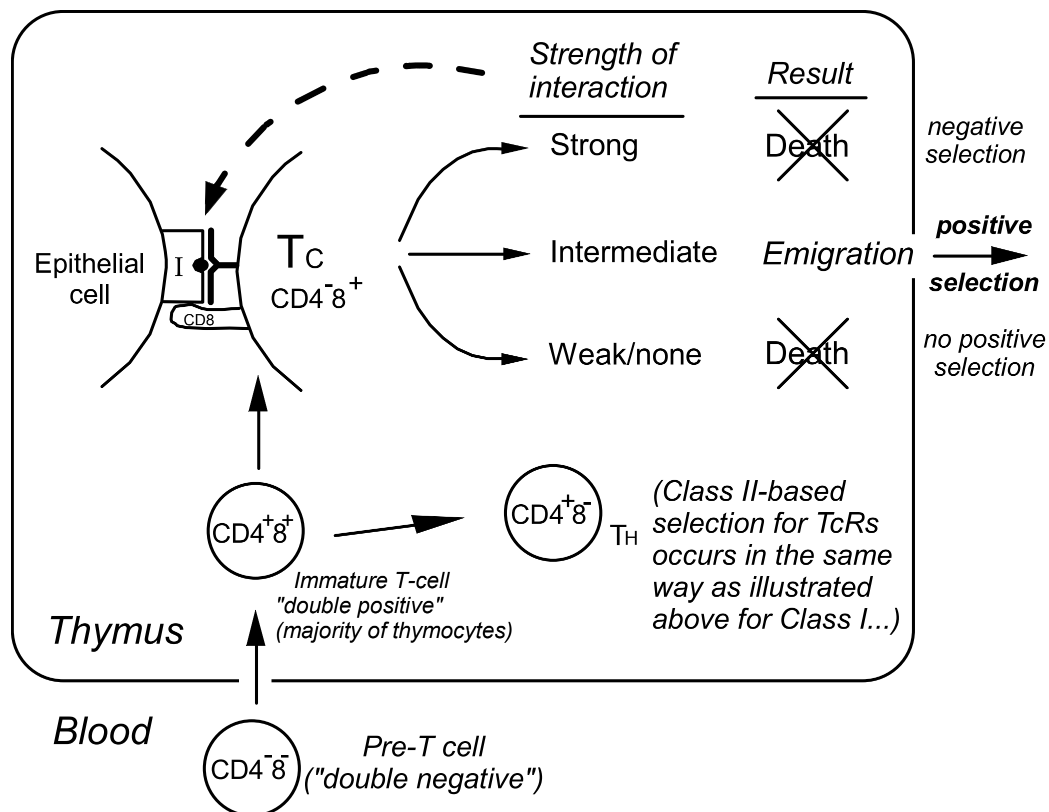


Figure 18-1

Bone-marrow-derived *pre-T-cells*, which bear neither CD4 nor CD8, enter the thymus from the bloodstream, develop into immature T-cells which are both CD4 and CD8 positive, then lose one or the other marker to become mature $CD4^+$ ("helper") or $CD8^+$ ("cytotoxic") effector cells. As part of this process, they undergo selection based on the interaction of their newly acquired T-cell receptors (TcR) with either Class I (for $CD8^+$cells) or Class II (for $CD4^+$) MHC expressed on epithelial or dendritic cells within the thymus. These MHC molecules are associated with a normal complement of "self" peptides. The case of a $CD8^+$

T-cell is illustrated in the figure, and is shown interacting with MHC Class I on an epithelial cell. If its TcR happens to interact *strongly* with a Class I molecule presenting some self-peptide, it is eliminated as a potentially auto-reactive cell; this process is termed *"negative selection"*, and is analogous to conventional "clonal abortion" which operates on developing B-cells. However, if this cell *never* encounters a Class I molecule in the thymus to which its TcR is capable of binding *at all*, then the T-cell is also eliminated, since it will not be able to carry out MHC-associated recognition of foreign antigens when necessary.

Only if the cell experiences a TcR/MHC interaction which is at some *intermediate* level (not too strong, not too weak), will the T-cell eventually be allowed to emigrate from the thymus as a mature $CD8^+$ T-cell; this is the process termed *"positive selection"*. It is among this successful population of T-cells, selected to recognize "self" MHC at some intermediate level, that cells exist which may *recognize a foreign antigenic peptide in self-MHC strongly enough to proliferate* to generate an immune response when triggered to do so (as we have discussed in Chapters 14 and 15). Although Figure 19-1 shows only a TcR/Class I interaction, a similar process of selection takes place within the thymus involving $CD4^+$ T-cells interacting with MHC Class II on dendritic cells.

Thus, Clonal Abortion (*i.e*. "negative selection") is a major contributor to establishing the normal state of tolerance among both B-cells and T-cells. Because this mode of tolerance is generated during the development of T-cells within the thymus (a "central" lymphoid organ), it is referred to as CENTRAL TOLERANCE. However, it is important to recognize that while this process of elimination of self-reactive clones *does* take place, it is not completely efficient. *In fact, T-cells and B-cells with receptors capable of recognizing and responding to self antigens can readily be found in the blood and lymphoid organs of normal individuals*. The recognition that clonal abortion is *not* a sufficient explanation for the normal state of self-tolerance led to the realization that *active* processes must be involved, including the process referred to as SUPPRESSION. The generation of immunological suppressor or "regulatory" cells, as well as a variety of other mechanisms which can maintain tolerance by processes taking place outside the thymus (outlined below), are collectively referred to as PERIPHERAL TOLERANCE.

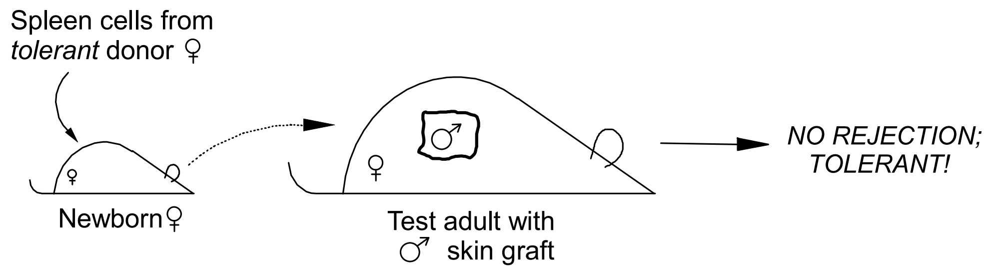
TOLERANCE MAY BE CAUSED BY ACTIVE SUPPRESSION

Let's illustrate an example of tolerance mediated by "suppression" using the male-specific antigen of mice. The H-Y antigen, controlled by a gene on the Y-chromosome, is present on a variety of tissues in male mice, but not in females. In certain strains (C57Bl/6 for instance) this can be demonstrated by grafting skin from males onto females and observing that the grafts are rejected, although relatively slowly. Grafts in the opposite direction, from females onto males, are accepted permanently.

Female mice may be rendered *tolerant* to the H-Y antigen if they are injected soon after birth with spleen cells from male mice of the same strain (as expected from Owen's results discussed above). Such females will *not* reject male skin grafts as adults.

We can carry out a simple transfer experiment to test the hypothesis that this experimentally induced tolerance is due simply to clonal abortion, *i.e*. the elimination of clonally precommitted cells reactive to H-Y antigen. As illustrated in Fig 19-2, below, we transfer spleen cells from a *tolerant* female to a *normal newborn* female, in exactly the same manner

we did with male cells, then ask if the recipient female can subsequently reject male skin as an adult. If tolerance is due simply to clonal abortion, we should *not* induce a tolerant state in the recipient, since all we are transferring are cells *lacking* reactivity to H-Y. The recipient's own immune system should develop the normal number of H-Y reactive cells and should be normally responsive.



Transfer of tolerance to H-Y antigen

Figure 18-2

What we find, however, is that *the recipient mouse grows up to be tolerant to H-Y* and cannot reject male skin as an adult. Therefore, clonal abortion (even though it may indeed have occurred in the tolerized female) *cannot* fully explain the tolerant state, and cannot explain at all the *transfer of tolerance* as described above. We must hypothesize an *active* process which has become known as *SUPPRESSION*, carried out by cells known as *SUPPRESSOR CELLS*. More recent work has established that such "suppressor" function is carried out by a population of CD4+ T-cells originally referred to as suppressor T-cells (T_S), which are now more commonly referred to as "regulatory" T-cells (T_{reg}), and which act to prevent the maturation of antigen-precommitted T or B-cells into effector cells in an *antigen-specific fashion*. Research on the functions and diversity of T_{reg} cells has, in recent years, become an exciting and tremendously important area of cellular immunology. The mechanism(s) by which T_{reg} carry out their functions is not fully understood, although it is known to involve the production of inhibitory cytokines in response to antigenic stimulation.

IMMUNE REACTIONS REPRESENT A DYNAMIC BALANCE BETWEEN IMMUNITY AND SUPPRESSION

The immune system's response to any antigenic challenge can be regarded as being a dynamic balance between immunity and tolerance, as illustrated below:

Following any given antigenic challenge, the generation of *helper* cells pushes the system toward an effective immune response (both cellular or humoral) while the generation of *regulatory* cells pushes toward tolerance induction. The net result may be immunity, tolerance, or some balance between the two.

What are the factors which determine whether a particular antigenic challenge will result in tolerance, immunity or neither? While the mechanisms involved in these processes are not

fully understood, a fair bit is known about the phenomenology, as illustrated in the table below:

Influencing Factor	To Induce Immunity:	To Induce Tolerance:
● Nature of antigen	Aggregated, insoluble	Monomeric, soluble
● Dose of antigen	Moderate dose	Very *high* or *low* doses
● Route of immunization	Subcutaneous, intramuscular	Intravenous
● Adjuvant	Use of adjuvant	No adjuvant

Table 18-1

While none of these factors is absolute in its behavior, this table outlines those features which are generally considered when one is attempting to generate a particular kind of immune response. Note that the general properties of *adjuvants* (Freund's, for example) maximize the induction of immunity while minimizing the induction of tolerance; Freund's converts a *soluble* antigen into *insoluble* form (by creating a water-in-oil emulsion), and it moderates the effects of high doses of antigen by releasing it over a period of time.

CLONAL ABORTION: EXPERIMENTAL MODELS

We have already discussed two experimental examples of tolerance induction *in vitro* by the mechanism of Clonal Abortion in Chapter 7. Ada and Byrt were able to eliminate antigen-specific clones by incubation of precursor cells with radioactive antigen, and Mishell and Dutton accomplished a similar end by killing proliferating cells with radioactive thymidine.

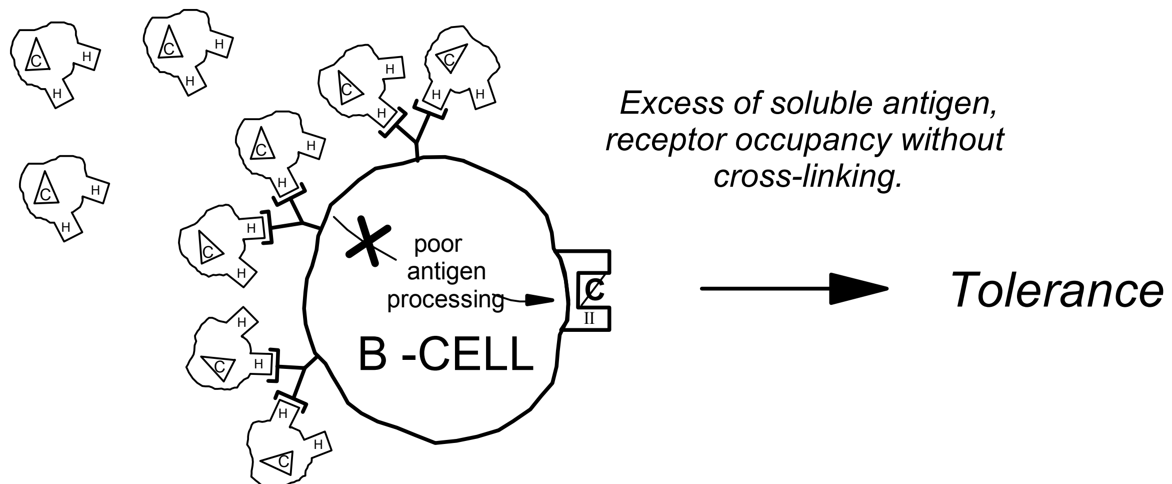
Effective clonal abortion can also be induced *experimentally* in whole animals by a variety of drugs which selectively kill proliferating cells. If the cytotoxic drug **Cytoxan** (cyclophosphamide) for instance, is administered to a mouse for several days following injection of SRBC, one finds not only that the mouse fails to make an antibody response to SRBC, but that it is *specifically unresponsive* to subsequent doses of this antigen for a period of several weeks. Those specific cells which have undergone clonal expansion in response to the initial dose of SRBC have been selectively eliminated by the drug treatment, effectively creating a state of tolerance by clonal abortion (as in the *in vitro* Mishell-Dutton experiments). It has not been possible to effectively adopt such treatments to humans, however, due to the severe toxicity of such drugs.

TOLERANCE BY RECEPTOR BLOCKADE

As indicated in the table above, a high dose of non-aggregated, soluble antigen tends to induce tolerance rather than immunity. We have already mentioned one example of this phenomenon in Chapter 2; injection of a high dose of *"clarified" BSA* into mice not only fails

to induce an immune response, but creates a *state of tolerance* to this antigen which lasts as long as the levels of circulating BSA are kept high. Part of the reason for this is that although such an antigen is very poorly processed by accessory cells (and therefore poor at triggering a T-cell response), it is capable of directly binding to the Ig receptors on B-cells without cross-linking these receptors. *Occupation of receptors on B-cells, without concomitant signaling by T-helper cells, paralyzes the B cells and renders them "**anergic**"*; not only do they fail to be triggered by this soluble antigen, but they are no longer capable of being triggered by subsequent appropriate T cell signaling so long as their receptors are occupied in this way. Thus, while these specific B-cells have not been killed they are anergic, and a state of tolerance exists so long as they remain unresponsive and have not been replaced by newly produced B-cells.

Figure 18-3 illustrates a hapten-specific B-cell which has its Ig receptors occupied, but not cross-linked, by an excess of soluble antigen. Compare this diagram with that in Figure 15-1. In the absence of MHC Class II presentation there is no T-cell help generated; furthermore, even if there were active T-helper cells available, such a B-cell would *not* be triggered, since cross-linking of its antigen receptors (*"Signal I"*) is a prerequisite for activation.



B-CELL TOLERANCE BY RECEPTOR BLOCKADE

Figure 18-3

Natural tolerance to at least some serum proteins may be maintained by this mechanism. For example, in certain patients who have very low natural levels of serum IgA, transfusion of exogenous IgA may result in an "autoimmune" anti-IgA response; this IgA is treated by the immune system as a foreign protein. This does not ordinarily happen in normal patients who receive blood transfusions, at least in part because potentially autoreactive B-cells are kept paralyzed by the normally high levels of circulating IgA.

ANTIGEN SEQUESTRATION AND "PRIVILEGED SITES"

An animal's normal lack of immune response to "self" antigens is not always the result of tolerance as define above. There are proteins in the eye lens and in spermatozoa, for

instance, which are highly immunogenic when injected into normal recipients (either male or female). Why, then, does an animal's immune system *not* normally make a response to these antigens?

It appears to be at least partially due to the fact that the immune system is not normally exposed to these antigens; they are *sequestered*, or localized in areas which have no lymphatic drainage. As a result there is *neither immunity nor tolerance* induction until the antigen is introduced to the immune system experimentally (or, as we will see later, accidentally [see Chapter 19]). Upon such exposure the immune system responds normally, even though the antigen is "self" and the resulting immune response can be highly damaging to the organism.

Areas within an organism which have no lymphatic drainage are referred to as **immunologically "privileged sites",** since antigens normally present or experimentally introduced into those areas do not generally stimulate an immune response. Thus, foreign tumor cells can be grown in the cheek pouch of a hamster, although in other locations they would be rapidly rejected. In humans, the eye, brain, testes and embryo have been recognized as immunologically privileged sites.

The immune protection of these "privileged sites", however, is never perfect, and antigen sequestration is never the sole contributor to the lack of immune responses to antigens in such regions. There also exists some degree of true peripheral tolerance mediated by T_{reg} cells, even for those antigens which are largely restricted to privileged sites.

CHAPTER 18, STUDY QUESTIONS:

1. Define “positive” and “negative” selection in the process of T-cell maturation within the thymus. Compare this maturation process with that of B-cells; in what ways are they similar or different?
2. Describe how you might attempt to induce a state of tolerance *in an intact animal* using each of three approaches: Hot antigen suicide or ^{3}HTdR suicide (see Chapter 7), or cytoxan-induced tolerance.
3. What might be some problems associated with carrying out each of the procedures from Question 2 in humans?
4. What are the differences between CENTRAL and PERIPHERAL TOLERANCE?

CHAPTER 19
AUTOIMMUNITY: BREAKDOWN OF SELF-TOLERANCE

A breakdown of the normal balance between immunity and self-tolerance can result in AUTOIMMUNITY, through the generation of an excessive level of T-cell helper function, a deficiency in the normal process of T-cell suppression, or an escape by autoreactive T-cells or B-cells from the normal process of clonal abortion. Several human diseases (and animal models) illustrate the key features of autoimmune states (THYROIDITIS, ENCEPHALOMYELITIS, MYASTHENIA GRAVIS, RHEUMATOID ARTHRITIS and SLE), and the significance of IMMUNE COMPLEXES in the pathophysiology of autoimmune disease.

An immune response to "self" antigens defines a state of **autoimmunity**. Clinically, autoimmune states may be mild or symptom-free, but in other cases may result in severe and fatal afflictions.

Autoimmune responses may result from two general causes. In one case, antigens normally "hidden" from the immune system (*"sequestered" antigens*) may be released into the circulation and trigger a response by the immune system. In this case no state of peripheral tolerance need exist (see Chapter 18), and the immune system is capable of generating an effective response on exposure to the antigen. Examples of clinical or experimentally induced autoimmunity to such sequestered antigens include those directed against *thyroglobulin, eye lens protein* and *spermatozoa* antigens. (Keep in mind, however, that sequestration of antigens within some anatomical compartment is never absolute, and that at least some degree of central and/or peripheral tolerance to such antigens is often demonstrable).

A different situation exists when the normally effective tolerant state of the immune system to "self" antigens is for some reason abrogated. The final result of such a *breakdown of tolerance*, however, is the same as in the case above: a damaging and potentially fatal autoimmune reaction.

Breaking of the normal tolerant state of an organism's immune system may result from a variety of influences:

i) Decrease in T_{reg} activity.

ii) Increase in T_H activity (*e.g.*, with adjuvants).

iii) Immune response to foreign antigens which happen to cross-react with "self" (for example, the experimental induction of rabbit anti-AChR, and streptococcal-induced rheumatic fever in humans)

The mechanisms which maintain a normal state of tolerance are certainly not 100% efficient, and we have already noted in Chapter 18 that T and B-cells with potentially autoreactive antigen receptors can be found in healthy individuals. *Low levels of autoantibodies can also*

be commonly found in humans and other organisms, much more frequently than one can find clinically significant autoimmune disease. There are, however, many clinical and experimental situations in which autoimmune processes play a key role in active tissue destruction and disease.

We discuss below a few examples of human autoimmune diseases and identify their key features. For some of them we will also present experimental autoimmune diseases which have been central to our understanding of the underlying immunological basis of the human diseases. We will discuss little of the details of clinical treatment of different human autoimmune diseases, but will note that it often includes generalized immunosuppression by steroids or other drugs.

HASHIMOTO'S THYROIDITIS. Autoimmune damage to the thyroid, primarily by the humoral response, results in decreased function and clinical *hypothyroidism*. It is characterized by the presence of circulating antibodies to thyroglobulin and thyroid peroxidase as well as to microsomal proteins and other components of thyroid cells. A mononuclear infiltrate is also typically present within the thyroid. The etiology of this disease is generally unclear, but may include initial damage to the organ from either a viral infection or trauma. Treatment includes hormone replacement, and, as with most autoimmune diseases, there is a significant genetic component which includes contributions by HLA linked genes.

> **AUTOIMMUNE THYROIDITIS OF MICE**
> When healthy mice are injected with homogenized mouse thyroid tissue together with complete Freund's adjuvant, they will develop autoantibodies to thyroglobulin as well as other antigens normally hidden inside cells of the thyroid. There will also develop a *cellular infiltrate* of the thyroid characterizing T-cell mediated immunity, and progressive damage to the organ resulting in hypothyroidism. This is considered an example of autoimmunity by deliberately induced *release of sequestered antigen* (despite the fact that low amounts of thyroglobulin are normally present in the circulation.)

GRAVE'S DISEASE. This is also an autoimmune thyroid disorder, but is characterized by *hyperthyroidism*. Autoantibodies are produced which are directed against the receptor for TSH (Thyroid Stimulating Hormone) which is expressed on thyroid follicular cells, and these antibodies stimulate the chronic overproduction of thyroid hormone. This is an example of an antibody acting as an *agonist* for its target molecules (*i.e.* they *stimulate* TSH activity), as opposed to an *antagonist* (*e.g.* anti-AcChR antibodies which *block* receptor function – see below). Treatment in such cases most commonly involves reducing thyroid function by surgery or radioactive iodine.

ACUTE DISSEMINATED ENCEPHALOMYELITIS (ADEM). This disease may follow infection by (or rarely vaccination with) a variety of viral pathogens, including measles, rubella and influenza. Cellular infiltrates representing a cellular immune response to myelin basic protein are evident, very similar to EAE, but without a humoral response. A wide variety of neurological defects can result from this autoimmune reactivity, which may be fatal.

MULTIPLE SCLEROSIS is another human disease characterized by inflammatory demyelination in the central nervous system (CMI involving $CD8^+$ T-cells specific for myelin basic protein, or MBP). Its causes are unknown, although the appearance of high levels of antibodies to measles and other viruses suggests a connection with viral infection.

> **EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE) OF MICE**
> Mice immunized with brain tissue or with the basic protein of myelin in Freund's adjuvant develop a progressive and often fatal degeneration of myelinated nerves. It is typically characterized by the presence circulating antibodies to myelin as well as mononuclear infiltration of nervous tissue. However, the disease can be transferred to healthy mice by adoptive transfer of immune lymphocytes, but not by transfer of serum, indicating that *only the cellular immune component is pathogenic*. As with experimental autoimmune thyroiditis, this represents an immune response to a normally sequestered antigen following deliberate breaking of normal tolerance by use of an adjuvant.

MYASTHENIA GRAVIS. This disease of unknown etiology is associated with *circulating antibodies* against the AcCh receptor, resulting in progressive muscle weakening. It can be treated with anti-cholinesterase drugs together with immunosuppressive therapy. An interesting histological aspect of this disease is the frequent presence of structures resembling germinal centers within the thymus (remember that the normal thymus contains neither B-cells nor germinal centers). Thymectomy is often beneficial in treating this disease for reasons which aren't fully clear, although cells producing anti-AcCH antibodies have been found within these ectopic germinal centers.

> **RABBIT AUTOANTIBODY TO ACETYLCHOLINE RECEPTOR (AcChR)**
> In the 1970's, researchers immunized rabbits with purified acetylcholine receptor protein from the ray, *Torpedo*, in order to produce antibodies to help in studying this molecule. Much to their surprise, the rabbits rapidly developed a progressive and fatal weakening of their muscles, which was found to be the result of autoantibodies directed against their own AcCh receptor molecules. This represents autoimmunity due to a *response to a cross-reactive antigen*; the rabbits' immune systems responded to the foreign protein of *Torpedo*, which happened to be closely enough related to their own receptor that it broke the normal state of tolerance.

Two other human autoimmune disorders are of particular importance, namely **Rheumatoid Arthritis** and **Systemic Lupus Erythematosus**. These are examples of a larger group of related disorders known as the *Rheumatic Diseases*, and are part of the group of **"non-organ-specific"** autoimmune diseases, to distinguish them from those considered **"organ-specific"** (which include the diseases discussed above).

RHEUMATOID ARTHRITIS (RA)

One common feature of RA is the presence of autoantibodies directed against the Fc fragment of IgG (as well as other "self" components, nuclear proteins and other intracellular molecules). The *anti-IgG autoantibodies are known as* ***rheumatoid factor***, and are mostly IgM (but may be IgG). The resulting immune complexes may be deposited in various sites within the vasculature and the joints. In either location they can cause tissue damage through

the fixation of complement and the attraction of PMN's; these processes result in the synovitis and vasculitis which (among other features) characterize this disease.

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

SLE is characterized by the presence of autoantibodies to a wide variety of autoantigens, including DNA, RNA, histones, ribonucleoproteins and other nuclear and cellular elements (often with *rheumatoid factor* present as well). These antibodies are associated with a pattern of damage to an equally wide variety of tissues and organs including skin lesions (erythema) and damage to the vocal cords which results in a characteristic hoarseness (*lupus* = "wolf"). In both RA and SLE (as well as many other autoimmune diseases), the etiological connection between the autoantibodies and the disease state is far from clear; did an initial breakdown of normal tolerance result in production of autoantibodies which cause the tissue damage, or did some other process initially cause tissue damage which then caused the subsequent breakdown of tolerance?

AUTOIMMUNITY AND IMMUNE COMPLEX DISEASE

We've already examined (in Chapter 5) the pathological consequences of circulating immune complexes, which can result in damage to the vasculature in general, and to the kidney in particular. It is important to remember that the **presence of circulating immune complexes is a common feature of many autoimmune diseases**, most notably the non-organ specific ones such as RA and SLE; these autoimmune complexes exhibit the same sequelae as those formed with heterologous antigens, *i.e.* fixation of complement and local tissue damage. The *kidney* is an especially important target of autoimmune-mediated damage. The presence of immunoglobulins on the basement membranes of the kidney is a diagnostic feature of distinct autoimmune states, depending on whether they show a *"smooth" distribution* (representing antibodies directed against the basement membrane itself, as in Goodpasture's syndrome) or a granular *"lumpy-bumpy" distribution* (representing the deposition of circulating antigen-antibody complexes, as in RA and SLE). We have also discussed in Chapter 5 the crucial role that complement plays in the normal removal of circulation immune complexes, and that defects in this clearance system may predispose to the development of autoimmune disease.

CHAPTER 19, STUDY QUESTIONS:

1. For three human autoimmune diseases, describe an underlying mechanism which is known from an animal model.

2. What relationships exist between the complement cascade and autoimmune disease?

CHAPTER 20
IMMUNODEFICIENCY

> CONGENITAL immunodeficiencies, while generally uncommon, serve to illustrate many of the key features of the development and function of the immune system, particularly those involving the CENTRAL LIMB. The ACQUIRED immunodeficiencies, particularly those of IATROGENIC origin, are much more common and are of broad clinical importance, fueling continuing searches for more effective and selective immunosuppressive drugs.

The critical importance of the immune system to our everyday health and well-being becomes especially obvious when one observes the results of deficiencies in immune function. These immunodeficiencies can be classified into two major categories:

CONGENITAL ("PRIMARY") IMMUNODEFICIENCIES - Victims are born with these diseases, which are the result either of *inherited* or *developmental* defects.

ACQUIRED ("SECONDARY") IMMUNODEFICIENCIES - These are *acquired* as secondary results of various disease states, due either to the disease processes themselves or the therapy used to treat them.

We can also classify immunodeficiency states with respect to which of the three "limbs" of the immune response is affected, that is whether they represent defects in the AFFERENT, CENTRAL or EFFERENT limb. Remember that the Afferent limb represents *antigen processing* carried out by macrophages and related cells, the Central limb involves the *triggering and proliferation* of clonally precommitted T-cells and B-cells, and the Efferent limb is the *effector* limb, involving the various effector T-cells (T_C T_{reg} T_{DTH} ...) and the biological consequences of antibody binding (which may include complement fixation, phagocytosis and allergic responses).

We will briefly go over a few examples of immunodeficiencies, keeping in mind that these are intended only as illustrations of the importance of various elements of the immune system. This list is far from complete, and the discussion of each example will cover only a few of its most striking features.

CONGENITAL DEFICIENCIES OF THE EFFERENT LIMB

CHRONIC GRANULOMATOUS DISEASE

Syndrome: Granulocytes and monocytes carry out their normal functions of phagocytosis, but are incapable of killing the organisms they phagocytose due to a deficiency of the enzyme NADPH oxidase, required to produce the "oxidative burst". Patients are susceptible to various microorganisms which are normally of low virulence, particularly with *Staphylococcus aureus* and gram-negative bacteria.

Inheritance: This disorder can be caused by several different genetic defects, one of which is controlled by an X-linked gene; symptoms appear at about two years of age.

Treatment: antibiotic therapy for infections.

COMPLEMENT DEFICIENCIES

A variety of genetic deficiencies of complement components are known, which we will not review here. Some may have only very mild consequences, but they are often associated with differing degrees of increased susceptibility to *bacterial infections*. Interestingly, deficiencies of some of the early complement components also appear to be associated with increased susceptibility to development of *lupus* and other *autoimmune states*, underlining the importance of the early components of complement in the normal process of *clearance of immune complexes* (discussed in Chapter 5).

CONGENITAL DEFICIENCIES OF THE CENTRAL LIMB

The congenital defects of T-cell and B-cell lineages are the most biologically interesting and informative of the immunodeficiencies, although the clinically significant ones are extremely rare. As we review four examples of such conditions, it will be useful to refer to Figure 21-1, which briefly outlines some key features of the process of differentiation of hemopoietic cells, focusing on the lymphocytic lineage.

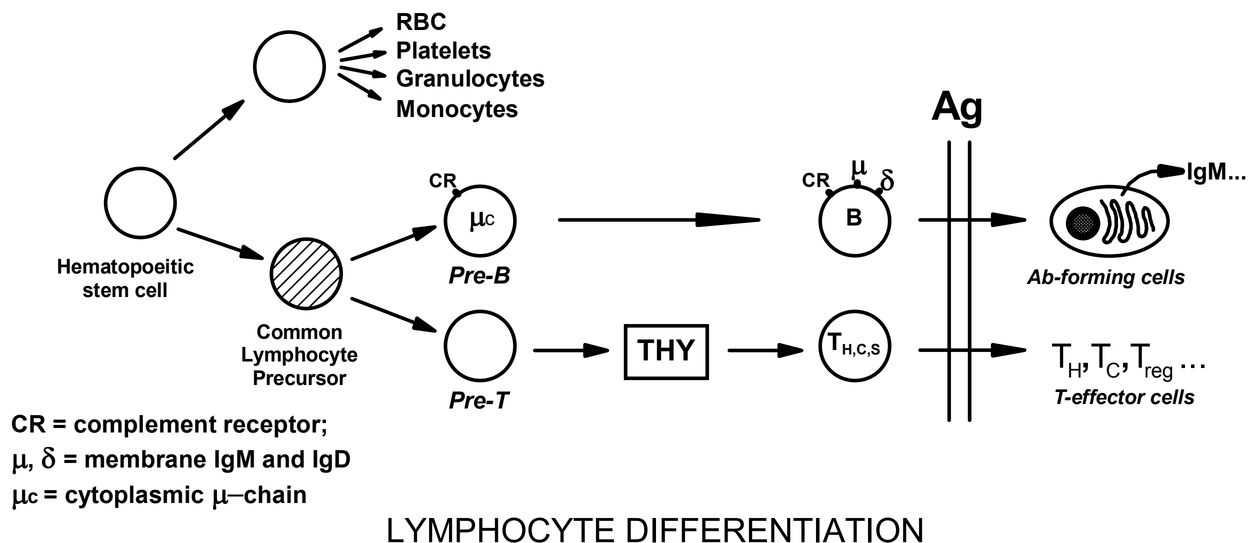


Figure 20-1

X-LINKED INFANTILE HYPOGAMMAGLOBULINEMIA (BRUTON'S SYNDROME)

Syndrome:

Extreme susceptibility to **bacterial infections** (more so than to viruses or fungi) starting at ~4-6 months of age.

Serum IgG is extremely low, other Ig isotypes absent.

B-cells (membrane Ig-bearing) are absent, but CR-bearing pre-B-cells may be present. Faulty Ig heavy chain gene rearrangements; D/J rearrangement may occur, but no V/D/J.

T-cell numbers and function are normal.

No follicles or germinal centers, or plasma cells.

Inheritance: X-linked recessive gene, frequency ~1 in 10^5. This gene has been identified as encoding a tyrosine kinase ("Bruton's Tyrosine Kinase", or BTK) which is expressed selectively in developing B-cells.

Treatment: Includes chronic treatment with gamma-globulin (*intravenous* IgG for passive immunization) and antibiotics.

This genetic defect prevents B-cell precursors from developing into functional B-cells; replacement therapy with human IgG is very effective. Since the development of IV-Ig, these patients have been surviving well past their fourth decade. Nevertheless, live vaccines are dangerous for these patients and should be avoided.

CONGENITAL THYMIC APLASIA (DiGEORGE SYNDROME)

Syndrome:

Hypocalcemic tetany is evident within 24 hrs. of birth (due to a deficiency of PTH, or parathormone, which is normally produced by the parathyroid and regulates potassium and calcium metabolism).

Repeated infections with **viruses and fungi**, (also bacteria); *Candida* and *Pneumocystis carinii* are characteristic.

No functional thymus (hypoplasia or aplasia).

Few or no T-cells.

B-cells present, but with variable function (serum Ig levels are also variable).

Primary follicles present in lymphoid tissues but without germinal centers, and empty thymus-dependent areas.

Inheritance: This disease is most commonly sporadic (not inherited) due to *de novo* deletion of a region of chromosome 22q11 (remember the distinction between "congenital" and "inherited"). This deletion results in a *developmental defect* of the 3rd and 4th pharyngeal pouches which give rise to both the thymus and parathyroid. Often associated with malformations of the heart and face, DiGeorge is considered as one manifestation of the larger complex of diseases collectively known as *cardiovelofacial syndrome*. The frequency of the deletion is ~1 in 10^4, the appearance of severe immunodeficiency is less common.

Treatment: Successful treatment may be carried out with **fetal thymus transplant**; the transplant need not contain lymphocytes, only a small amount of thymic epithelium. Any therapy must consider the danger of GvH reaction; for this reason one must not use adult thymus tissue, and any transfused blood must be first X-irradiated. No *live* vaccines should be given.

This disease is the result of the absence of T-cell development through failure to provide an appropriate environment for differentiation. The patient's lymphocytes themselves (pre-T cells) are perfectly capable of developing into mature cells, and if the appropriate environment is provided (by a thymic transplant) they will colonize it and develop normally.

SEVERE COMBINED IMMUNODEFICIENCY (SCID)

Syndrome:

Overwhelming infections in first year of life.

No functional T- or B-cells (abnormal B-cells may be present).

Thymus and peripheral lymphoid tissues empty or severely depleted of lymphocytes.

Inheritance: *Several different known genetic defects* can cause this disease, including both X-linked and autosomal recessive forms, with an overall frequency around 1 in 10^6

Treatment: The only successful treatment is replacement of the hemopoietic stem cells by bone marrow transplant. Prevention of GvH is a key to success (by removing cells of the T-cell lineage).

This genetic defect is expressed in the *common lymphocyte precursor* of T and B-cells indicated in the diagram above, and prevents their further development. If a source of competent stem cells is provided (*e.g.* by bone marrow transplantation), they will colonize the thymus and peripheral lymphoid tissues in a normal fashion.

SELECTIVE IgA DEFICIENCY

Syndrome:

Very low serum levels of IgA (<0.05 mg/ml), normal levels of other isotypes.

Normal cell-mediated immunity.

Increased susceptibility to viral and bacterial sinopulmonary infections, although it may often be asymptomatic. Can also be associated with autoimmune or allergic states.

Inheritance: Genetic basis variable and poorly understood; frequency up to 1 in 300.

Treatment: No specific treatment other than antibiotic therapy. IgA should not be administered, as it can trigger an anti-IgA autoimmune or allergic responses (see Chapter 21).

This is the most common of several isotype-specific Ig deficiencies; it is not known whether it is a defect in the precursors of IgA-producing cells, or in the (unknown) mechanism by which their differentiation is regulated.

ACQUIRED IMMUNODEFICIENCIES

A. SECONDARY TO DISEASE

1) Many **infectious diseases** result in more or less general immunosuppression. In the case of Human Immunodeficiency Virus (HIV), the agent which causes Acquired Immunodeficiency Disease (AIDS), its pathogenicity is a direct consequence of its severe depression of immune responsiveness.
2) **Malignancies** can often result in immunosuppression, either by generally interfering with normal physiological functions, or through the production of factors which specifically suppress immune functions (this may be a significant aspect of the natural biology of malignancy).
3) **Renal failure** can cause the loss of large amounts of serum immunoglobulins into the urine, resulting in humoral immunodeficiency.
4) **Enteropathies** can lead to loss of immunoglobulin through the gut, with similar results.

B. IATROGENIC. *("Caused by the healer"*; referring to a condition which is the result of therapeutic treatment.)

This category includes *the most common immunodeficiency conditions* which most physicians will encounter. They are the result of various forms of therapy which have either as their goal or as a major side effect the suppression of immune responsiveness. Increased susceptibility to infections is an important consequence of immunosuppression which must always be considered, and balanced against the therapeutic benefits of a particular treatment. Some therapeutic treatments which can have this result are:

1) **Corticosteroids**. Prednisone is widely used for both its anti-inflammatory effect and immunosuppressive capability; lymphocytes in general are very sensitive to steroids.
2) **Cytotoxic drugs**. Many anti-tumor drugs (such as azathioprine and cyclophosphamide) are strongly immunosuppressive as well, and may also be used intentionally for this purpose. Susceptibility to infections may therefore be a major side effect of anti-tumor therapy, in a patient who may already be immunosuppressed by the presence of the tumor itself.
3) **Anti-Lymphocyte Antibodies**. Sera from horses immunized with human thymocytes contain effective anti-T-cell antibodies, and have been used extensively since the 1950's to inhibit rejection of transplanted organs; such preparations are known as Antilymphocyte Serum (ALS) or Anti-Lymphocyte Globulin (ALG). Their use is much more limited now than in the past, as a result of the increasing availability of more selective monoclonal antibodies (see Chapter 14 and APPENDIX 13) and the development of new classes of immunosuppressive drugs (such as cyclosporin and FK-506, mentioned below).

4) **Ionizing Radiation**. X-rays or gamma-rays, often used in tumor therapy, also destroy whatever lymphoid tissue happens to be in their path. While ionizing radiation generally kills proliferating cells in a highly selective manner (which is the basis for its anti-tumor effectiveness), *resting lymphocytes are unusually sensitive to such radiation*.

CYCLOSPORIN A AND FK506 (*Tacrolimus*)

Since the early 1980's Cyclosporin A, a fungal peptide, and FK506, a bacterial macrolide, have served as remarkably effective immunosuppressive agents in human transplantation. They inhibit T-cell function to a greater extent than B-cells or phagocytic cells, selectively blocking T_H cell function by interfering with the production of and response to IL-2, but without killing the cells. While they are less effective in inhibiting *established* immune responses, they have nevertheless been tremendously useful in prolonging kidney, liver and heart transplants. Their molecular target, however, is not restricted to cells of the immune system, and they exhibit a variety of often serious side effects, including nephrotoxicity. Although they have not completely replaced steroids and cytotoxic drugs in clinical immunosuppressive therapy, cyclosporin and FK506 have allowed the use of lower doses of these drugs, resulting in far less severe generalized immunosuppression and other side effects in the management of transplant recipients.

CHAPTER 20, STUDY QUESTIONS:

1. For each of the congenital immunodeficiency diseases discussed, identify the relevant biological defect and describe how it leads to the symptoms of the disease.
2. What situations may result in iatrogenic immunodeficiency states?

CHAPTER 21
IMMEDIATE HYPERSENSITIVITY: ALLERGY

Allergies represent TYPE I reactions according to the *Gell and Coombs* classification. Most are caused by IgE ANTIBODIES which are capable of binding to Fc-receptors for IgE on tissue MAST CELLS. Cross-linking of these membrane-bound IgE's by specific antigen results in mast cell DEGRANULATION; this process releases HISTAMINE and a variety of other effector molecules, which in turn results in the myriad symptoms of allergy. (rash, hay fever, asthma *etc*.) Passive cutaneous anaphylaxis (PCA) in the guinea pig and the Prausnitz-Küstner (P-K) skin reaction in humans provide models for understanding the underlying mechanism of allergic reactions. Management of allergies begins with allergen avoidance, and includes the use of a variety of drugs and allergen-specific DESENSITIZATION.

One of the many manifestations of antibody, specifically antibody of the IgE isotype, is the mediation of **allergic reactions**. We are all familiar with such reactions, whether they appear as hay fever, asthma, sensitivity to penicillin or other drugs, or skin reactions due to substances in food or cosmetics. This class of humoral immune reactions is referred to as **immediate hypersensitivity**, since the time required for its development is much shorter than typically required for cellular immune reactions (**delayed-type hypersensitivity**) or other kinds of humoral reactions.

Any substance which can elicit an allergic response is referred to as an ALLERGEN. However, an allergen can only be effective in causing an allergic reaction if the recipient has been previously sensitized; that is, there must be present, in his tissues, antibodies of the IgE class directed against the allergen. An allergen can be a component of any number of microorganisms, plants or animals, or may be a synthetic compound. Allergic reactions may be elicited by exposure through the air (pollen), contact with the skin (cosmetics), ingestion (natural or artificial food products), or injection (drugs or insect bites).

GELL AND COOMBS CLASSIFICATION OF IMMUNE REACTIONS

Before describing the mechanisms involved in allergic reactions, we will review a general scheme of classification of immune reactions proposed almost fifty years ago by Gell and Coombs. While this scheme is quite dated in many respects, it remains widely used and can be helpful in understanding the relationships between different immune reactions,

Under this scheme, all immune reactions are classified into one of four headings known as Types I, II, III, and IV.

Type I reactions include all of **immediate hypersensitivity**, the allergic reactions we will cover in detail in this chapter. They are mostly IgE-mediated (although other Ig classes may sometimes participate), and their rapid onset, typically within minutes of exposure to antigen, is characteristic.

Type II reactions result from **antibody binding to membrane-bound Ag** resulting in complement-mediated cytotoxicity or opsonization/inflammation. This class of reactions may be typified by hemolytic anemia (resulting from auto-antibodies directed against red blood cells), hemolytic disease of the newborn (*HDN*, see Chapter 10), and certain drug reactions.

Type III reactions occur when Ab binds to *soluble* Ags to form **immune complexes,** which can cause any of several kinds of immune complex disease (see Chapter 5). While IgG, IgM and complement may be involved, as in Type II, the key difference is that *Type III reactions are the result of deposition of circulating antigen-antibody complexes in tissues, as opposed to the binding of antibodies to antigens which are an integral part of a target cell membrane*.

Type IV reactions include all **cell-mediated reactions** (Cell-Mediated Immunity or CMI). **Delayed-type hypersensitivity** (DTH) is a synonym for CMI, and alludes to the slower development of such reactions compared with antibody-mediated reactions. For example, the tuberculin skin test to PPD (Purified Protein Derivative) develops over a period of a few days, compared with a few minutes for Type I reactions and a few hours for Type III.

GELL AND COOMBS CLASSIFICATION OF IMMUNE REACTIONS

Reaction	Description	Participating Antibody	Skin Reaction Example (Cellular Infiltrate)	Typical Time of Onset
TYPE I	Immediate hyper-sensitivity (*e.g.* allergy)	IgE (plus Mast Cell)	Allergy skin test (eosinophils & PMNs, *late phase* only)	1-20 min
TYPE II	Ab to cell-associated antigens (*e.g.,* hemolytic anemia)	IgG/IgM	----	---
TYPE III	Ab to soluble Ags, immune complexes (*e.g.*, serum sickness)	IgG(IgM)	Arthus reaction (neutrophils [PMNs])	7-10 hrs
TYPE IV	Delayed-type hypersensitivity (DTH); Cell-mediated immunity (CMI) (*e.g.* graft rejection)	---	Tuberculin test (mononuclear cells, *i.e.* lymphocytes and macrophages)	1-3 days

These distinct kinds of immune reactions can be distinguished not only by the *time course* of their development, but also by the nature of the *cellular infiltrate* present in the sites of a typical reaction. We have seen (Chapter 5) that one of the consequences of complement fixation is the release of factors which are chemotactic for PMN's; thus we typically find these cells in Type III reactions (Arthus reaction). As we will see later in this chapter, one of

the mediators of allergic reactions is a factor which is chemotactic for eosinophils--thus the characteristic infiltrate of the *late phase* of Type I reactions (*e.g.* allergy skin test). The mechanisms of cell-mediated immunity have been discussed in Chapter 12, and we have seen that lymphocytes and macrophages are both effector cells of such reactions, and that macrophage-chemotactic factors are known mediators. Thus, *mononuclear* cells (a term encompassing macrophages and lymphocytes) characterize the inflammation at the site of a tuberculin skin test (Type IV reaction). We can therefore see a clear connection between the nature of the cellular infiltrate identified by the pathologist, and what is known of the cellular and molecular mechanisms of immune reactions.

IgE: REAGINIC ANTIBODY

IgE antibodies are also known as **reagins** or **reaginic antibody**. They have several features which distinguish them from other classes of Ig:

Low serum levels — IgE serum concentrations are lower than any other class of Ig, typically in the range of nanograms per ml. (But remember that only *IgE which is bound to mast-cells* can initiate allergic reactions.)

Heat labile — IgE is very sensitive to heat, and can be inactivated by moderate heating which has little effect on other Ig classes (for instance, heating at 56C for 30 minutes, typically carried out to inactivate complement).

"Homocytotropic" — This refers to the ability of an animal's Ig to bind to its *own* mast cells and initiate allergic reaction. IgE is the major mediator of allergy, although in humans IgG4 can also carry out this function with lower efficiency. [This term exists only to distinguish IgE from *"heterocytotropic"* antibody, which can initiate allergic reactions only in a species other than the one from which the antibody was obtained. Rabbit IgG, for instance, can cause allergic responses when transferred to guinea pigs, although it does not do so in the rabbit. Heterocytotropic antibodies are mostly of experimental interest.]

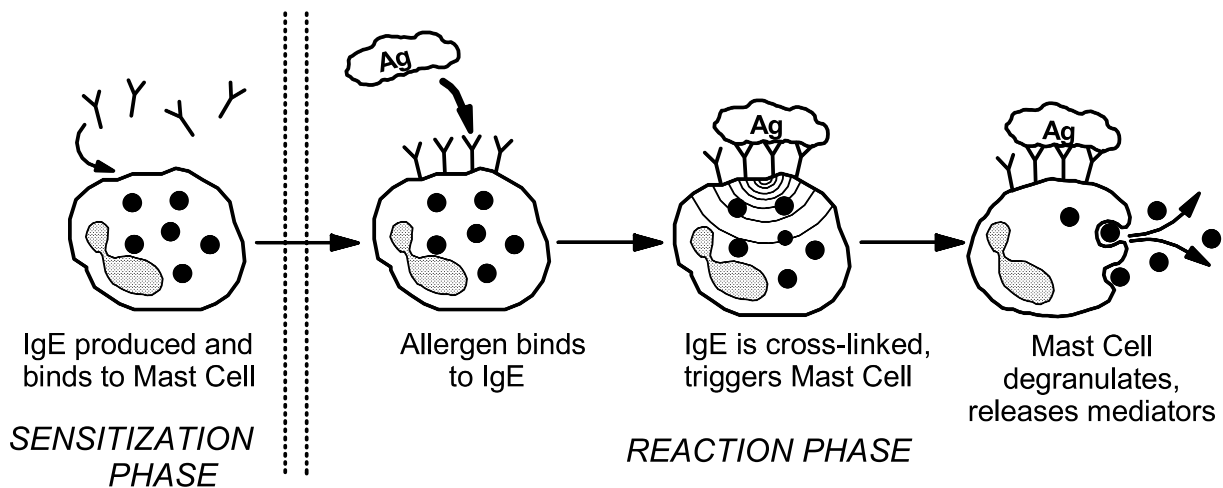
SEQUENCE OF EVENTS IN ALLERGIC RESPONSES

Allergic immune responses can be separated into two components, a period of *sensitization* followed by the allergic *reaction* itself.

Sensitization Phase

1) Exposure of the immune system to the immunogenic allergen results in the **induction of IgE antibody**, with the participation of T-cells, B-cells and macrophages. This induction of Ig synthesis must precede the allergic reaction itself, which is outlined in Figure 18-1.

2) **IgE antibody binds to tissue mast cells** through its Fc piece (see Figure 18-1). Mast cells have Fc receptors on their surface with an extremely high affinity for IgE antibodies. The stage is now set for the initiation of an allergic response.



IgE AND ALLERGIC REACTIONS

Figure 21-1

Reaction Phase

1) Re-exposure to the allergen results in the **binding of the allergen to mast-cell-bound IgE antibody**. Cross-linking of IgE molecules on the mast cell surface by multivalent antigen is required for the subsequent reactions.

2) The large, basophilic granules present in the mast cells are released into the tissues; this process of **degranulation** releases a variety of pharmacologically active compounds; some directly from the granules (e.g. histamine and heparin), others newly synthesized by the cell soon afterwards (see below).

Mediator	Effect
Histamine, heparin, proteases	Contraction of the smooth muscle of bronchi, gut and venules; capillary dilation & vascular permability; increased mucus secretion
Prostaglandins, leukotrienes,	Bronchoconstriction; asthma; increased mucus secretion and venule permeability.
Kinins	Vasodilation, histamine-like activity
Cytokines, chemokines, interleukins (IL-4,8)	Inflammation, tissue remodeling
ECF-A *(Eosinophil Chemotactic Factor ofAnaphylaxis)*	Eosinophilia

These and other active compounds can lead to such conditions as **asthma** and **rhinitis**, generally in response to allergens in pollen, dust or animal dander. Allergens in cosmetics can lead to **rashes**, and allergens in food, drugs or injected animal poisons may result in rashes or in **vascular** or **gastrointestinal anaphylaxis**. These conditions may range from very mild to severe, and are potentially fatal (for instance, in the case of severe asthma or vascular anaphylaxis).

The nature and severity of allergic reactions depends on the route of exposure and the degree of sensitization, it varies considerably from one species to another, and within humans may vary tremendously between different individuals; there are clearly very significant genetic factors which are not understood, but they include at least some HLA- linked components.

EXPERIMENTAL MODELS OF ANAPHYLAXIS

Vascular Anaphylaxis in the Guinea Pig

The *guinea pig* is extremely sensitive to the development of severe allergic reactions, and was for many years the system of choice for studying allergic responses and the physiology of IgE.

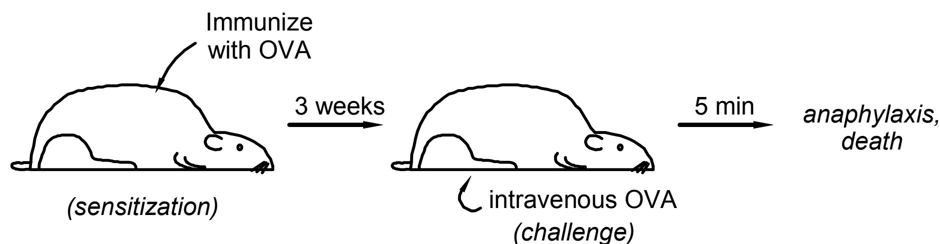


Figure 21-2

A guinea pig can be sensitized by intramuscular injection of an antigen, say OVA (ovalbumin). Its immune system responds by producing antibody to OVA, including (but not exclusively) IgE. Some of this circulating IgE will be fixed onto mast cells in various tissues, including the vasculature and respiratory tract. Three weeks later, the same animal can be challenged either with an intravenous dose of OVA or by exposure to an aerosol containing OVA. Following IV injection, the animal will rapidly develop severe vascular shock and die within a few minutes (the combination of *venule constriction* and *capillary dilation* results in *pooling* of blood in the peripheral circulation and a drastic drop in blood pressure). If exposed to the aerosol, it will equally rapidly die from bronchial constriction, an experimental model for human asthma.

Passive Cutaneous Anaphylaxis (PCA)

The ability to carry out an anaphylactic reaction can be transferred from a sensitive animal to a normal one by transferring serum (this defines it as a humoral immune response). This can

be done in such a way as to cause a local rather than systemic reaction, as illustrated by PCA (Figure 18-3). Serum is collected from a guinea pig sensitized to OVA (as described above), and a small amount is injected intradermally into a normal recipient.

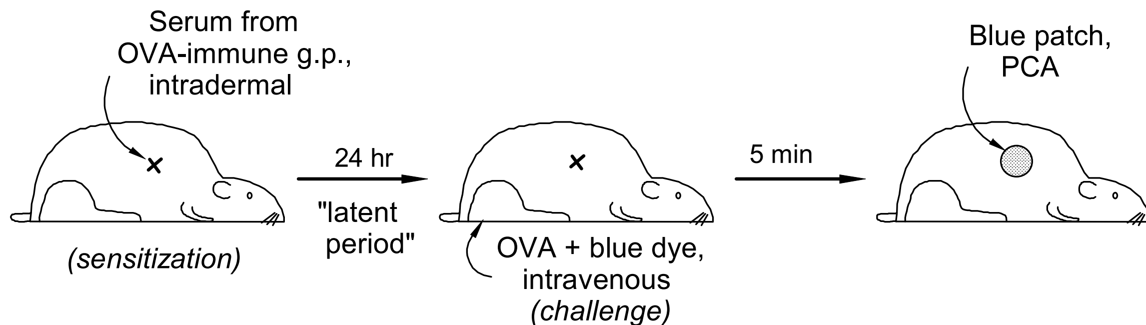


Figure 21-3

After 24 hours (the **latent period** required for the IgE antibodies to bind to the surface of local mast cells), OVA together with the dye Evans Blue is injected intravenously. Within minutes, a blue patch becomes visible on the animal's skin where the immune serum had been injected. This is because local degranulation of the mast cells has resulted in capillary dilation, and the leakage of fluids from blood into the intercellular spaces of the skin is readily visible due to the presence of blue dye.

Prausnitz-Küstner (P-K) Reaction

This is a human version of passive cutaneous anaphylaxis, and is primarily of experimental rather than routine clinical interest. Serum from an "atopic" (allergic) patient is injected intradermally into a non-allergic recipient. Twelve hours later (this is the **latent period**) a small amount of the allergen is injected into the same region. Within minutes the allergic reaction becomes visible as a **wheal and flare**. The "wheal" is a region of swelling resulting from the leakage of fluid into the extracellular space (edema), the red "flare" surrounding it is the result of increased blood flow.

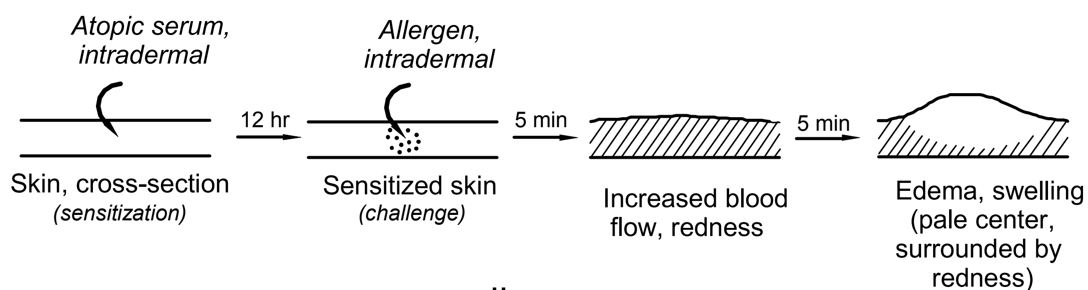


Figure 21-4

EARLY-PHASE *VERSUS* LATE-PHASE ALLERGIC REACTIONS

Allergic reactions are referred to as "Immediate Hypersensitivity" because of their typically rapid onset. However, there are often important long-term consequences of the immediate reaction which are collectively referred to as the *Late-Phase Reaction*. In the case of an allergic skin reaction (Prausnitz-Küstner, for example) the early or immediate phase occurs within a few minutes. However, over the course of subsequent hours and days a cellular infiltrate dominated by eosinophils will develop, which also includes neutrophils, macrophages and TH2 cells (keep in mind that we have only mentioned a few of the many inflammatory and chemotactic factors released by mast cells during degranulation).

In the case of asthmatic reactions, the late-phase response may be manifested by a chronic inflammation and hypersensitivity of the bronchi, with important diagnostic and therapeutic implications. Cromolyn sodium, mentioned below, can be given prophylactically to inhibit mast-cell degranulation and prevent the initial reaction. In the presence of an ongoing chronic late-phase reaction, however, administration of steroids may be indicated for their anti-inflammatory properties.

EVALUATION OF THE ALLERGIC STATE

In managing allergic patients, it would be useful to have a simple and accurate laboratory test to determine the specificity and severity of any allergy. While no ideal test exists, one can monitor IgE levels in such patients, as well as skin sensitivity to defined antigens, with varying degrees of usefulness.

Determination of serum IgE levels in allergic patients can be carried out by several assays. One of them, the RadioImmunoSorbent Test (RIST), measures total *serum IgE*; however, these levels correlate very poorly with the degree of clinical allergy. Somewhat better is the RadioAllergoSorbent Test (RAST), which measures the levels of circulating IgE *antibody* directed against a particular allergen; still, the correlation is not good.

The reasons why these tests are less than satisfactory are not fully clear, but reflect the fact that there exists a high degree of variability among different people, not only in the levels of IgE (relative to other isotypes) in humoral immune responses, but also in the efficiency of its fixation to tissue mast cells, the sensitivity of mast cells to degranulation, and the sensitivity of various tissues to the effector molecules and the inflammatory response.

Skin testing can be very useful in determining to which allergens a particular patient is sensitive. This involves injecting small amounts of various purified antigens intradermally, and measuring the severity of the *wheal and flare* reaction over a period of about half an hour. (This is an *active* rather than a passive cutaneous anaphylaxis reaction).

Test diets are also very helpful in identifying food allergies. The patient is placed on a diet free of the most common sources of allergens (wheat and related grains, dairy products, eggs, *etc.*), and various foods are added one at a time over a period of weeks or months to determine which may be responsible for the allergic response.

TREATMENT OF THE ALLERGIC STATE

1) **Avoidance** of allergens is the single most important and effective element in managing allergic states. This may include eliminating known allergens from one's diet (nuts, milk, eggs, for instance), removing them from one's home (wool carpets, feather pillows, pets and plants, for instance), and, of course, avoiding drugs to which one is sensitive (penicillin and its derivatives, for instance). Eliminating the major known allergens from one's environment can not only avoid reactions to those substances, but can also reduce overall sensitivity to other allergens.

2) **Desensitization** may be quite effective, but not in all cases. This involves deliberate immunization with small but increasing amounts of a particular purified allergen over a period of months or years. It is thought that the effectiveness of this procedure results from the induction of high levels of IgG antibodies, which can prevent allergic reactions by competing for the allergen and preventing it from reaching mast-cell bound IgE.

   Desensitization has been useful for pollen and dust allergens as well as for some animal danders, but its effectiveness is unpredictable and varies between individuals. It is not generally useful for food or drug allergies (although a protocol has recently been introduced for penicillin allergies). Desensitization also carries with it a certain degree of risk, as each injection may itself induce a more or less severe allergic reaction. The procedure must be carried out under careful supervision.

3) **Drugs** can be useful in both preventing and treating allergic reactions.

   Antihistamines inhibit binding of histamine to its receptors (but will not effect the target tissue response once histamine is bound).

   Corticosteroids are anti-inflammatory (useful for the late-phase response), and can also inhibit histamine synthesis.

   Cromolyn sodium is widely used as a prophylaxis for excercise and allergy-induced asthma, although its mechanism of action is unclear. While it is known to stabilize mast cells, this is probably not the basis for its efficacy.

   Isoproterenol, salbutamol and epinephrine can be used to counteract the effects of various of the mediators released by mast cells; *unlike the drugs mentioned above, they can be effective in controlling an ongoing allergic reaction*. Prompt treatment of a systemic anaphylactic reaction (to a food, drug or insect bite, for example) can be life-saving.

PENICILLIN SENSITIVITY: SELF-PROTEINS MAY ACT AS CARRIERS

Type I allergic reactions to penicillin are not uncommon, and can be life-threatening. The mechanism of generation of such reactions is of interest. The beta-lactam ring of this antibiotic is chemically highly reactive, and can spontaneously and covalently bind to serum proteins. The result is, in effect, the production of a hapten-carrier complex which may be immunogenic (despite the fact that the carrier molecules are "self"); they are capable of

generating a humoral antibody response (including IgE) to the penicillin hapten, and of triggering an allergic reaction once IgE is present. (Penicillin bound to red blood cells may also serve as a target for antibody-dependent cell destruction and hemolytic anemia, an example of a "Type II" immune reaction.)

Other drugs and chemicals can also bind to "self" proteins, and *give rise to allergic reactions (humoral immunity) in addition to delayed type hypersensitivity (cell-mediated immunity)* to small molecules which would not by themselves be immunogenic. DTH skin reactions to metals such as nickel in jewelry are generated in this manner, producing *Contact Dermatitis*.

WHY IgE?

While the dangers and discomfort associated with IgE-mediated reactions are well known, the physiological benefits derived from IgE are less clear; surely the evolution of the IgE system did not occur in order for us to enjoy the benefits of hay fever and asthma. One important clue seems to be the association of extraordinarily high levels of IgE (thirty or more times normal levels) with certain helminthic infections, and IgE-mediated contraction of the smooth muscle of the gut may obviously promote expulsion of large numbers of the parasites.

It has also been shown that eosinophils can cause damage to some of these parasitic worms, but the situation remains puzzling. Most of the excess IgE is not specific for the parasites, and the eosinophilia associated with such infections affords only a limited level of protection. In short, the damaging effects of the IgE system are still understood far better than the positive role it must have had in mammalian evolution.

CHAPTER 21, STUDY QUESTIONS:

1. Is the IgE antibody bound to a particular mast cell monospecific? Is it monoclonal?
2. Would heating an antiserum at 56C for 30 minutes affect its ability to transfer an ARTHUS reaction? A PRAUSNITZ-KŰSTNER REACTION? CONTACT DERMATITIS?
3. What is the rationale for allergy DESENSITIZATION?

CHAPTER 22
VACCINATION

VACCINATION has had a huge impact on increasing human life expectancy. ACTIVE IMMUNIZATION confers long-lasting immunity by inoculation with *killed* or *attenuated organisms, toxoids or purified antigens*. PASSIVE IMMUNIZATION by transfer of antibodies has the advantage of more rapidly establishing protection, but it is short-lived and may carry a risk of inducing *serum sickness*. The route and schedule of immunization, physical nature of the vaccine antigen, and immune status of the host all contribute to the relative effectiveness of any vaccination protocol, in ways which are still not fully understood.

HISTORY

Awareness of acquired resistance to infectious diseases has existed since ancient times. This knowledge led to the development in China, several centuries ago, of the technique of **variolation**, the deliberate inoculation of small amounts of material from smallpox pustules into healthy individuals. Having recovered from the disease induced by this treatment, which *generally* was milder and less dangerous than the naturally acquired disease, the inoculated individuals had a life-long immunity to smallpox. Variolation was brought from China to the West, and was increasingly widely used in Europe and the New World through the eighteenth century. It remained, however, a dangerous procedure with an appreciable risk of death, and provided a source of infection for spread to others.

This technique was replaced almost overnight following Jenner's publication of the results of his technique of **vaccination.** He had learned of the fact that milkmaids who had handled cows which were suffering from **cowpox** developed a high degree of resistance to the related human disease, smallpox. He then was able to show that inoculation of cowpox material into humans, while not causing serious disease, was in fact capable of producing resistance to smallpox.

The distinction between *variolation* and *vaccination* is an important one. In *vaccination*, the fact that the inoculated material is *not* a virulent human pathogen makes this technique much safer than variolation, and it derives its name from the species which originally provided this material (*vaccus* = cow).

MODES OF IMMUNIZATION

A) ACTIVE IMMUNIZATION

Active immunization, of which Jenner's original vaccination is an example, involves the inoculation of immunogenic material to induce an immune response (and therefore immunological memory) in the recipient. *Active immunity takes longer to develop* than passive (see below), but also lasts much longer, and may often be life-long.

Various forms of antigenic material may serve as a vaccine:

i) **Killed organisms**. The viruses which cause *rabies, influenza* and *polio* (the latter in the case of the Salk vaccine) can be collected, killed by treatment with heat or chemicals, and used as effective vaccinating agents. The bacteria responsible for *cholera, whooping cough (pertussis)* and *typhoid fever* can be used in the same manner.

ii) **Attenuated organisms**. Live viruses, but in a weakened or "attenuated" form, provide effective vaccination for *measles, mumps* and *polio* (Sabin vaccine), and more recently for influenza (in a form administered by nasal mist). Attenuated bacterial vaccines also exist, typified by those for *anthrax* and for *tuberculosis* (*BCG*, "Bacille de Calmette-Guerin", an attenuated form of the organism which causes bovine turberculosis). The advantage of attenuated organism over killed ones is that they can set up active (although hopefully harmless) infections and provide more effective stimulation of protective immune responses.

Attenuation of viruses may be achieved by growth *in vitro* in the laboratory and selection of genetic variants with limited pathogenic potential. While the use of vaccinia (cowpox virus) also falls into the category of "attenuated viruses", it actually represents the fortuitous existence in nature of a *cross-reactive but non-pathogenic* organism. Similarly, attenuation of bacteria may be achieved by allowing bacterial cultures to "age" during laboratory culture with or without deliberate selection of variants.

A key factor in producing any viral vaccine is the development of a cell culture system which allows the growth of the virus in the laboratory. The development of the Salk vaccine in the 1950's, for example, followed hard on the heels of the discovery that monkey kidney cells in culture could be used to grow the virus.

iii) **Toxoids**. In the case of *diphtheria* and *tetanus* infections, the real danger in the disease comes not from the presence of the organisms themselves but from the potent toxins which they produce. Effective immunity can be induced by immunization with chemically modified toxins, or *toxoids*, which are no longer toxic but still highly immunogenic (and, of course, cross-reactive with the native toxins).

iv) **Purified antigens (*"subunit vaccines"*)**. Vaccines for *meningococcus (Neisseria meningitidis), pneumococcus (Streptococcus pneumoniae)* and the *Hepatitis B* virus each consist of purified antigens from these organisms, polysaccharide for the first two and protein for the last. In those cases where effective antigens can be identified and purified they have an advantage over attenuated vaccines in not posing any risk of infection, and over killed organism vaccines in being less likely to cause severe inflammatory reactions.

A relatively recent development in subunit vaccines can overcome the limitations of immune responses to carbohydrates, which are typically restricted to IgM. Carbohydrates can be chemically coupled to immunogenic protein carriers, and the resulting **conjugate vaccines** have been developed for pneumococcus and meningococcus (among others) which can stimulate stronger responses showing class switching and greater memory, and are effective in younger children.

v) **“Naked” DNA**. This relatively recent development in vaccination comes from the surprising finding that direct inoculation of DNA encoding a protein results in a strong and long-lived immune response to that protein, both humoral and cell-mediated. It is thought that the DNA transfects local APCs resulting in expression of the encoded protein in the context of both MHC Class I and Class II. One attractive feature of such a protocol is that it can yield a strong cell-mediated response without having to resort to the use of live virus vaccines. However, DNA vaccination in humans has not yet progressed past the clinical trial stage.

B) PASSIVE IMMUNIZATION

Injection of *antibody* to a pathogen can provide very *rapid*, although *short-lived*, resistance to infection, and is referred to as **passive immunization**. Passive immunization is generally used when there is no time to wait for the development of active immunity (see below), or when no effective active vaccine exists.

i) **Human antibodies**. Normal human IgG, prepared from pools of many individual donors, contains significant levels of antibody to *measles* and *hepatitis* viruses. High levels of protective antibody for *tetanus* can be obtained from immunized donors, and anti-*Zoster* antibodies from the serum of patients collected during recovery from an infection. In each case, these antibodies can be administered to recipients who are at high risk for acquiring the disease.

ii) **Heterologous antibodies**. Horse antibodies to *diphtheria toxin* or to the toxins of *snake and spider* venoms have been very effective in neutralizing the effect of these dangerous molecules. The use of heterologous serum, of course, introduces a substantial risk of inducing *serum sickness* or an *allergic reaction*.

C) ADOPTIVE IMMUNITY

We have already discussed *adoptive immunity* in experimental systems, illustrated by the transfer of immune reactivity to non-immune (and/or irradiated) recipients using *immunocompetent cells*, typically spleen cells. Obviously, such adoptive transfer of reactivity cannot be carried out in humans due to histocompatibility barriers, and adoptive immunity essentially does not exist in human medicine. This term is sometimes applied in a somewhat different context, *i.e*. describing the hematopoietic restoration/replacement which results from bone marrow transplantation, as, for example, in the case of Severe Combined Immunodeficiency (see Chapter 20).

SOME FACTORS AFFECTING IMMUNIZATION

i) **Adjuvants** (see also Chapter 2). The most effective experimental adjuvant known, Freund's Adjuvant, cannot be used in clinical medicine because of its severe side effects (inflammation, pain, and fever). Some human vaccines can be rendered more effective, however, by precipitating the antigen together with an aluminum hydroxide salt, a procedure known as *alum precipitation*; diphtheria toxoid is used in this form. Synthetic adjuvants usable in humans are currently being developed, some involving synthetic versions of the biologically active molecules of Freund's (including *muramyl dipeptide*).

In some cases one can take advantage of the natural adjuvant properties of certain vaccines, notably *pertussis*. The so-called *triple vaccine*, (DPT), consists of alum-precipitated **d**iphtheria toxoid, killed **p**ertussis organisms and **t**etanus toxoid. In this case, the pertussis organisms act as an adjuvant (much as *Mycobacterium* does in Freund's), which increases the immune response to the two purified protein antigens.

ii) **Route of immunization**. While most vaccinations are introduced through the skin, either by scarification (*e.g.* smallpox) or by ***injection*** (*e.g.* Salk polio vaccine and many others), the *Sabin polio vaccine* is one notable exception. The attenuated viral organisms are administered ***orally***, and they set up a chronic infection in the gut, stimulating a local IgA response. Since the normal mode of entry of polio virus is through the gut, this antibody response is precisely in the place where it should do the most good. (It should be noted, however, that the Sabin vaccine is no longer recommended for use in the U.S.) Similarly, one form of the influenza vaccine (Flumist) is administered as an ***intranasal mist***, mimicking the normal route of entry of the infectious organism.

iii) **Dose of antigen**. The dose and time course of human vaccinations is largely determined empirically; whatever works is used.

iv) **State of the host**. The effectiveness of active immunization naturally depends on the ability of the host to mount a normal immune response. It can be dangerous, however, to introduce any *live* vaccine into a host with a T-cell deficiency, since even an attenuated organism can give rise to a lethal infection in such an environment.

In the case of an immunologically healthy host, the degree of urgency may determine if passive or active immunization is appropriate. For tetanus, *active* immunization with the toxoid is generally used and is effective for ten years or more. However, in the case of a very severe wound, or a tetanus-prone wound which is several days old before being presented to the physician, *passive* immunization may be administered for immediate protection, together with active immunization for longer-lasting immunity. Similarly for rabies, passive immunization may be added to the standard active immunization in the case of a particularly severe rabies-prone wound, or one close to the head (since the brain is a major target of the virus).

OTHER ISSUES ASSOCIATED WITH IMMUNIZATION

A) **Antigenic variation**. Influenza virus, for example, can rapidly alter its antigenic structure by mutation, so that it is no longer recognized by antibodies made against the original virus. Influenza can therefore give rise to repeated infection with variants of the same organism.

B) **Antigenic competition**. Two antigens given at the same site can sometimes each interfere with the immune response to the other. In general, therefore, different immunizations are given at different times and/or at different sites. But remember that DPT is a notable exception to this rule, and other routine multiple vaccination protocols exist (such as MMR).

C) **Maternal immunoglobulin**. The presence of specific antibodies at the time of vaccination may interfere with its success. Measles vaccine, for example, should not be given before 15 months of age, since the presence of maternal IgG antibodies may prevent active immunization (remember RhoGAM, discussed in Chapter 10).

ACTIVE IMMUNIZATIONS

Diseases	Vaccine Type	Disease Organism
General		
*Poliomyelitis	killed virus (Salk)	Polio virus
	attenuated virus (Sabin) *[not currently recommended]*	
Measles } *"MMR"*	attenuated virus	Measles virus (Rubeola)
Mumps } *"MMR"*	attenuated virus	Mumps virus
Rubella *(German Measles)* } *"MMR"*	attenuated virus	Rubella virus
*Diphtheria } *"DPT"*	toxoid	*Corynebacterium diphtheriae*
*Pertussis *(whooping cough)* } *"DPT"*	killed bacteria	*Bordatella pertussis*
*Tetanus } *"DPT"*	toxoid	*Clostridium tetani*
*Hepatitis B	purified protein (recombinant)	Hepatitis B virus
Pneumococcal (PCV)	conjugated carbohydrate	*Streptococcus pneumoniae*
Meningitis	conjugated carbohydrate	*Neisseria meningitidis*
Hib	conjugated carbohydrate	*Hemophilus influenzae type b*
*Influenza	killed virus (injected)	Influenza virus
	attenuated virus (nasal mist)	
Varicella *(chicken pox)*	attenuated virus	Varicella-zoster virus
Special		
*Smallpox	live virus (Vaccinia)	Smallpox virus (Variola)
Rabies	killed virus	Rabies virus
Yellow fever	attenuated virus	Yellow fever virus
Typhus	killed organisms	*Rickettsia typhi*
Typhoid fever	killed or attenuated bacteria	*Salmonella typhi*
Paratyphoid	killed bacteria	*Salmonella paratyphi*
Cholera	killed bacteria	*Vibrio cholera*
Plague	killed bacteria	*Yersinia pestis*
Tuberculosis	attenuated bacteria (BCG)	*Mycobacterium tuberculosis*
Rocky Mountain Spotted Fever	killed bacteria	*Rickettsia rickettsii*
Shingles	attenuated virus	Varicella-zoster virus

**Key examples*

CHAPTER 22, STUDY QUESTIONS:

1. Describe at least one example each of clinical vaccination by (a) live organisms, (b) killed organisms, or (c) purified antigen. What are the advantages and disadvantages of each?
2. What are the benefits and risks of *active* versus *passive* immunization? Give examples.
3. How might one account for the phenomenon of *antigenic competition*?

CHAPTER 23
TUMOR IMMUNOLOGY

While the importance of the immune system's role in normally preventing the development of tumors is questionable at best, it *is* clear that immune responses against tumors often do develop, and can be detected and studied in both experimental and clinical situations. This chapter introduces some of the concepts required to understand the complex relationship between the immune system and tumor development, and a selection of the complex terminology which has characterized this field. Three key points are: (1) *Tumor cells often do express new antigens* ("neo-antigens") which are potential or actual targets for immune recognition. (2) Despite this fact, *the immune system is often ineffective* in eliminating tumors or preventing their growth. (3) Various means may be possible for *modifying anti-tumor immune responses* to render them more effective, and to use *immunological approaches for both diagnosis and treatment.*

NEOPLASIA:

Appearance of a *tumor* (from the Latin word for "swelling") results from ABNORMAL PROLIFERATION of cells, through the loss or modification of normal growth control. Cells which normally do not divide (*e.g.* muscle or kidney cells) may start proliferating, or cells which normally do proliferate (*e.g.* basal epithelial cells or hemopoeitic cells) may begin dividing in an uncontrolled fashion.

If the growth of a tumor remains localized, it can often be removed surgically (if it is accessible), and is therefore relatively harmless; or "benign". In some cases, however, cells from a growing tumor may be capable of intruding into adjacent normal tissue ("*invasive growth*"), and may leave the original site and begin to proliferate in a new location (METASTASIS). These properties distinguish "MALIGNANT" tumors from benign ones.

It is important to understand and reconcile two seemingly contradictory properties of tumors namely their MONOCLONAL ORIGIN *versus* their HETEROGENEITY. While tumors are almost invariably of *monoclonal origin*, mutation and chromosomal instability commonly generate a substantial degree of *heterogeneity* within tumor populations (see IMMUNOMODULATION and IMMUNOSELECTION, below). This fact has important implications for both diagnosis and treatment of cancers.

ORIGINS OF NEOPLASIA:

At least three major factors are known to initiate tumor development; regardless of which of the three is the cause (or if the cause is unknown), the consequence is uncontrolled growth, resulting either from the abnormally high expression of genes which *stimulate* cell proliferation (ONCOGENES), or defective expression of genes which normally *control* proliferation (TUMOR SUPPRESSOR GENES):

1) CHEMICAL CARCINOGENS: Many natural as well as man-made compounds present in our foods and environment are known to be capable of inducing tumors, *i.e.* they are *carcinogenic* (tobacco smoke and char-broiled steaks, for example, both contain complex mixtures of carcinogens).

2) IONIZING RADIATION: X-rays, gamma rays and ultraviolet (UV) radiation can all induce the appearance of cancers by their ability to cause changes in gene expression; We are exposed to these through sunlight (UV), normal background radiation (gamma rays), and clinical sources (gamma rays and X-rays).

3) VIRUSES: Many viruses carry their own *oncogenes*, *i.e.* genes whose products can stimulate neoplastic transformation of the infected cell; these genes may be expressed during a viral infection, and in some cases may be integrated into the genome of an infected cell and be expressed at a later time.

TUMOR-SPECIFIC ANTIGENS (TSA) and TUMOR-ASSOCIATED ANTIGENS (TAA):

As mentioned above, many tumors can be shown to express cell surface antigens which are not expressed in the normal progenitor cells before the neoplastic transformation event. These antigens have been categorized based on their nature and distribution, resulting in a complex collection of acronyms, some of which are defined here:

1) Chemical or radiation-induced tumors each generally express a *unique* neo-antigen, different from other tumors induced by the same or different agent. These have been termed Tumor-Specific Transplantation Antigens, or **TSTA**.

2) Tumors induced by the same virus express antigens *shared* between different tumors. These consist of membrane-expressed *virally encoded antigens*, and have been termed Tumor-Associated Transplantation Antigens, or **TATA** (since they are not, strictly speaking, tumor "specific").

3) **Oncofetal antigens**: These are TATAs which are *more or less* selectively expressed on tumors, but are also shared with some normal fetal or embryonic tissues. Examples include *carcinoembryonic antigen* (CEA, shared with healthy fetal gut tissue), and *alpha-fetoprotein* (AFP, also present in the serum of healthy infants, but decreasing by one year of age). Expression of such antigens by tumors is thought to reflect their reversion to a less fully differentiated state.

IMMUNOLOGICAL SURVEILLANCE:

In its classical form, this term refers to the idea that an important role of the adaptive immune system, particularly cell-mediate immunity (CMI), is to destroy newly appearing neoplastic cells as a result of the new antigens they tend to express. *However, considerable experimental evidence has failed to support this idea*, at least in its simplest form**.** Most importantly, immunosuppressed hosts do *not* generally show the expected high frequency of tumors predicted by this theory, a phenomenon which has been most strikingly illustrated in the *nu/nu* thymus-deficient mouse model, extensively studied for precisely this reason. Those

tumors which *have* been found more frequently in immunosuppressed hosts are usually those induced by viruses, and are best understood simply as the defective immune system's inability to control the viral infection as it normally would. The importance of the adaptive immune system in providing natural protection against neoplasia is therefore highly questionable at best, although NK ("natural killer") cells are still considered as possibly playing such a role. Nevertheless, as we will see, there has been considerable interest in exploiting those anti-tumor immune responses which are known to occur, and direct them toward more effective cancer therapy.

FACTORS WHICH LIMIT ANTI-TUMOR IMMUNE RESPONSES:

If tumors express antigens which can be recognized by the immune system, why are they not normally eliminated, as if they were a foreign tissue graft? Several more or less well-characterized processes have been shown to contribute to the ability of tumors to continue to grow despite the possible or demonstrated presence of a potentially destructive immune response.

TOLERANCE: A state of immunological tolerance may be promoted by the presence of very high levels of tumor antigens, particularly soluble forms which may be shed into the serum by tumor cells (see also Blocking Factor, below).

IMMUNOMODULATION/IMMUNOSELECTION:

Immunomodulation: Antibody binding to a membrane antigen on either normal or neoplastic cells may result in the disappearance of that antigen from the cell surface either by endocytosis (internalization) or by shedding. Once a tumor cell has lost its neoantigen, it becomes invisible to the immune system.

Immunoselection: Variant cells can appear in a tumor population which have lost a particular TATA or TSTA through a mutational event; such a variant will have a selective advantage in the face of an even slightly effective immune response (either humoral or cellular), and cells with the antigen-negative phenotype will therefore tend to progressively take over the population. Note that such a process depends on the presence of *heterogeneity* in a tumor cell population.

ENHANCING ANTIBODIES, IMMUNOSTIMULATION.

Enhancing antibodies: Remember that most nucleated cells (other than lymphocytes, which constitute an important exception) are relatively resistant to complement-mediated lysis, and destruction of grafted tissue is largely the role of cell-mediated immunity. However, if antibodies are present which can bind to neo-antigens, they may effectively "hide" these antigens which might otherwise serve as targets for T-cell-mediated killing, and thus "enhance" the survival of such cells.

Immunostimulation: Antibodies binding to tumor cells may also, in some cases, actually *stimulate* cell growth, presumably through the generation of receptor-mediated proliferative signals. We have previously discussed the fact that antibody directed against a cell surface receptor may act as an agonist with respect to the normal signaling pathway for that receptor (see Chapter 19, AUTOIMMUNITY).

BLOCKING FACTORS: Soluble factors have been described in the serum of tumor-bearing animals as well as in patients which can inhibit an existing immune response from affecting the tumor. The best characterized "blocking factors" have turned out to consist of circulating antigen-antibody complexes containing tumor antigens, which may blind or divert the immune response in ways which are still poorly understood.

IMMUNOSUPPRESSION: Tumor-bearing animals and human tumor patients often exhibit a substantial degree of generalized immunosuppression, resulting directly or indirectly from inhibitory cytokines and other substances secreted by the tumor (Hodgkin's lymphoma is one notable example).

CONCOMITANT IMMUNITY: A mouse bearing a progressively growing tumor may reject a new inoculum of the same tumor at a different site; this rejection is a manifestation of "concomitant immunity", an immune rejection reaction occurring at one site, co-existing with the progressive growth of an antigenically identical tumor elsewhere in the organism. This phenomenon illustrates the importance of *tumor mass* in determining the ultimate outcome of a battle between a tumor and the immune system; a small focus of tumor cells is more susceptible to immune killing than a large and well-vascularized tumor.

ACTUAL AND POTENTIAL APPLICATIONS OF IMMUNOLOGICAL PRINCIPLES TO THE CANCER PROBLEM:

IMMUNODIAGNOSIS

1) *Tumor-associated antigens in serum*. Detection of TSTA/TATA in serum to detect the presence of tumors which are undetectable by conventional methods, or to measure the overall tumor mass and its response to therapy. Assays for determining serum levels of CEA and PSA ("prostate specific antigen"), for example, are widely used for both screening and evaluation of the success of therapy.

2) *Antibody-based therapy*. Use of radioactively labeled antibodies to TSTA, to allow detection and localization of otherwise invisible metastases. While the use of radiolabeled antibodies for such purposes has been proposed and studied for decades, it has not yet advanced to routine clinical application.

IMMUNOTHERAPY

1) **Humoral**: A number of monoclonal antibodies which target tumor antigens have been FDA-approved for therapy, currently representing the most widely used form of immunotherapy. Many are used as unconjugated "naked" antibodies, and their effectiveness derives either from their ability to target tumor cells for destruction by fixing complement and opsonization, or by serving as antagonists for cell surface receptors important for cell proliferation or angiogenesis (growth of blood vessels).

Several decades of research have been devoted to the potential use of anti-tumor antibodies conjugated with toxins (*e.g. diptheria toxin*, or the plant toxin *ricin*), or highly radioactive isotopes, thus serving as targeting agents to deliver these toxic molecules to tumor cells. Only two conjugated mAbs, both radioactively labeled, have thus far been approved for human use as therapeutics, and are being studied for use in imaging (to detect otherwise invisible centers of growth). A number of other radioactive mAb conjugates are in various stages of development.

2) **Cell-mediated** (Lymphokine-Activated Killer [LAK] cells). Lymphocytes removed from a tumor-bearing patient can be treated *in vitro* with lymphokines (*e.g*. IL-2), and the resulting "activated" cells re-injected into the patient, in the hope that the tumor cells will be killed by tumor-specific killer T-cells present in the transfused cell population. These lymphocytes can be either obtained from the patient's blood (which hopefully contains some tumor-specific T-cells), or else extracted from a surgically excised tumor ("tumor-infiltrating leukocytes" or "TILs"), presumably consisting of a more highly enriched tumor-specific population. Results in humans have not been as positive as in experimental animals, but attempts continue to developing this approach into an effective therapeutic.

CHAPTER 23, STUDY QUESTIONS:

1. Define the concept of *"Immunological Surveillance"*. What is the strongest evidence regarding its validity?
2. Define and give examples of the terms *"Tumor-Specific Antigen"*, and *"Tumor-Associated Antigen"*.
3. Describe at least three mechanisms by which tumors are known to avoid immune destruction.
4. Describe *two* possible approaches to using antibodies for anti-tumor therapy.

APPENDIX

1. PRECIPITIN CURVE

Let's examine a typical ***precipitin curve,*** in which we take a *fixed* amount of antigen (say BSA), and add to it *increasing* amounts of antibody. We then measure the quantity of the resultant **precipitate**.

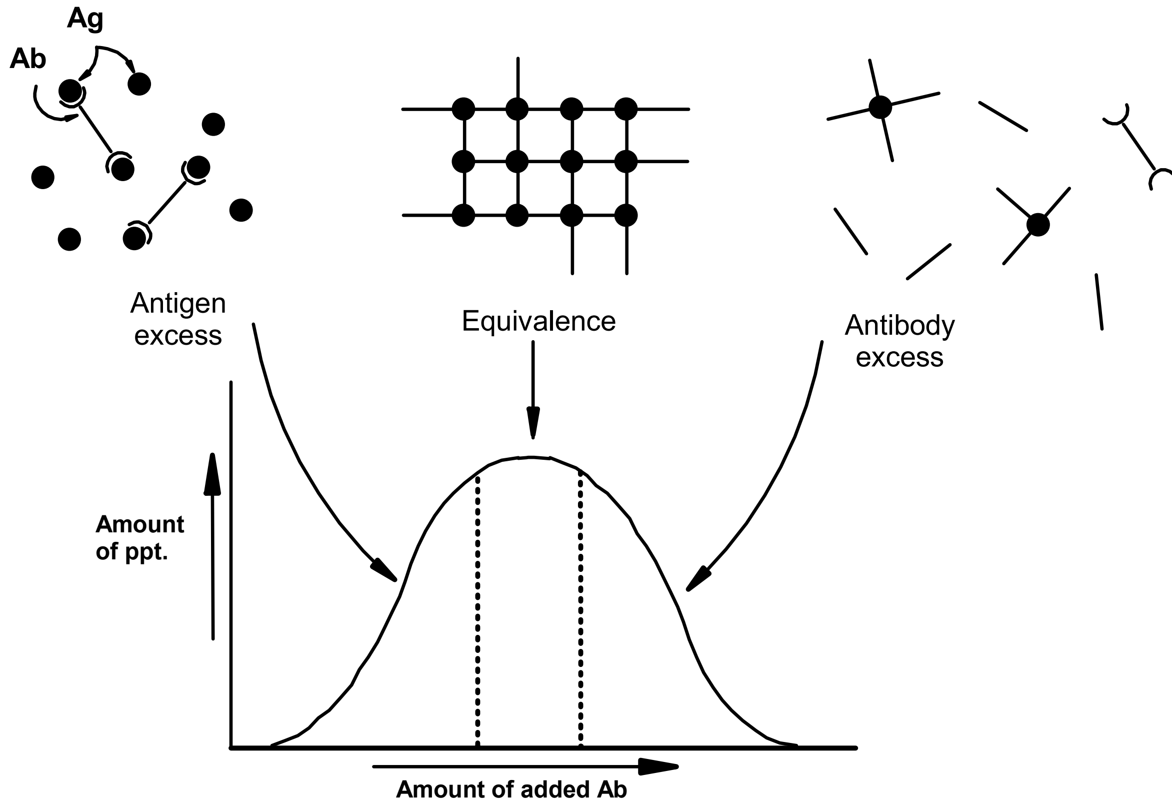


Figure A1-1

We find that the precipitate increases as we add more antibody (as expected), but that after a certain point the total precipitate *decreases* again. This is due to the formation of *soluble complexes* of two different kinds, one in the region of large antigen excess, the other in region of large antibody excess; these are illustrated in the diagrams above the precipitin curve. The antibody-antigen ratio near the peak of the curve is known as the **region of equivalence,** which results in the *maximum* formation of large, insoluble complexes. Since the binding of antibody with antigen is a *reversible* process, changes in the concentration of either component can drive the reaction in one direction or the other.

2. LABELING OF ANTIBODIES (AND OTHER PROTEINS)

The ability to label proteins with radioactive markers led to the development in the 1950s of the RIA (RADIOIMMUNOASSAY), which provided an extremely sensitive approach to detecting and quantitating antibodies and antigens. Other methods for labeling proteins have also been widely used, including fluorescent dyes and enzymes.

Radiolabeling - RIA. While radioactive isotopes of iodine (^{125}I and ^{131}I), are not the only radioactive markers used for proteins (^{3}H tritium and ^{35}S sulfur have also served, among others), they have been the most widely used. A variety of different reaction schemes can be used to attach either free iodide or small iodine-containing molecules to antibodies, resulting in a highly radioactive but still biologically active antibody molecule. These radioactive antibodies can be detected in extremely small quantities, either in precipitates or bound to a solid state antigen. For many years RadioImmunoAssays (RIA, see APPENDIX 5) have served as sensitive and widely used assays in both clinical and research settings. Beginning in the 1970's, however, RIA's have progressively been replaced by equally sensitive and less expensive Enzyme-Linked Immunoadsorbent Assays (ELISA, see below, and APPENDIX 5).

Fluorescent labeling - IF. Fluorescent dyes can be coupled to antibodies, and the binding of such labeled antibodies to bacteria (or other particulate antigens), or to antigens in histological sections, can be readily detected by fluorescence microscopy, a technique known as IMMUNOFLUORESCENCE (**IF**). The two classical dyes used have been Fluorescein (which fluoresces green) and Rhodamine (red), although a large number of newer dyes such as Texas Red and others have since been added to this list.

One major feature of IF is its ability to determine the localization of antibodies and antigens within histological sections; such studies were the first, for example, to demonstrate that plasma cells were the major site of antibody production. More recently, IF has been a major contributor to the power of **Fluorescence-Activated Cell Sorters (FACS),** machines which can analyze and separate complex populations of cells, *one cell at a time*. The basis of such separation is often the labeling of one or another cell type in a complex mixture with a fluorescent antibody specific for that cell type, but cells can also be sorted on the basis of size, DNA content and a variety of other physical and biological parameters.

Electron-dense labels. A variety of electron-dense markers can be attached to antibodies, including Ferritin (which has an extremely high content of iron atoms) and colloidal gold particles. The resulting antibodies can be visualized in the *electron microscope* just as fluorescent antibodies can localized by light microscopy, but, of course, with much higher resolution.

Enzyme labeling - ELISA. A variety of enzyme molecules can be coupled to antibodies, which can then be sensitively detected by their associated enzymatic activity. The resulting *ELISAs* (ENZYME-LINKED IMMUNOSORBENT ASSAY) are very widely used, and have replaced RIA's in most modern applications. Commonly used enzymes have included horseradish peroxidase, alkaline phosphatase, , β-galactosidase, and, more recently, luciferase.

ELISA's can equal or exceed the sensitivity of RIA's, and have the major advantage of not requiring the use of radioactivity with its associated costs and hazards. The detection method often involves the addition of a colorless substrate which the enzyme converts into a colored (or fluorescent) product, and the results can be tabulated photometrically in fractions

of seconds per sample as opposed to minutes per sample for typical RIAs. Another detection method which has become increasingly widely applied involves the use of enzymes which cause their substrates to emit photons of visible light ("luminescence"). Such *luciferase/luciferin* based assays can be considerably more sensitive than the more traditional colorimetric assays.

Enzyme-based histochemistry. Enzyme-labeled antibodies can also be used for microscopy in the place of fluorescent-labeled ones (see IF, above). In such cases a substrate is used which yields a visible and *insoluble* product which is deposited at the site of enzyme (and therefore antibody) localization. The fact that a single enzyme molecule can yield many molecules of product makes such **immunohistochemistry** potentially more sensitive than immunofluorescence, and the resulting microscopic preparation is more permanent and easier to photograph than fluorescent material.

3. OUCHTERLONY ANALYSIS

The Ouchterlony assay was widely used in myriad research and clinical contexts for many years after its development in 1948 , although it has been largely superceded by other assays which are more sensitive and suitable for quantitation (see APPENDIX 5). Nevertheless, this technique provides a very useful tool for illustrating and clarifying the principles of antibody heterogeneity and specificity, which is why we cover it in some detail here.

In the Ouchterlony assay, the antigen and antibody solutions are placed in nearby wells cut out of a thin layer of agarose, and allowed to stand for a few hours or a day or two. During that time they diffuse toward each other, and where they meet they will form a *visible line of precipitation*.

The pattern in which adjacent lines cross one another yields considerable information about the antigenic relationships between different antigens. Let's illustrate this with a pattern generated by a *rabbit antiserum made against whole human serum*, using three purified protein antigens as targets, namely HSA (*human serum albumin*), BSA (*bovine serum albumin*) and HTf (*human transferrin*). The well labelled "Ab" contains the antiserum, and the resulting pattern is shown below.

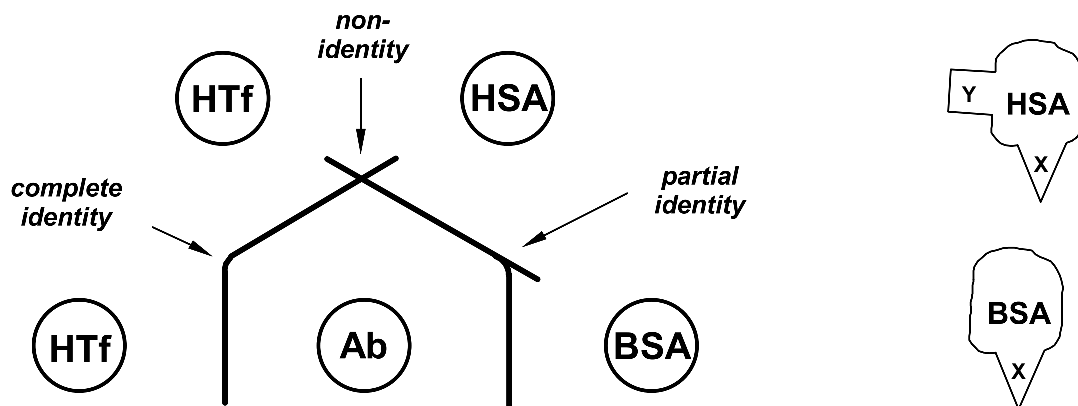


Figure A3-1

Several conclusions can be drawn from the pattern of precipitation shown here:

1) The antiserum (in the well labelled "Ab") contains antibodies against *all three antigens*, since each one shows a precipitin line.

2) The lines produced by the two adjacent wells containing HTf join completely, in a pattern of **complete identity,** or simply "*identity*." This indicates that the antigens in the two wells (which in this case we know are exactly the same) are *antigenically indistinguishable* by this antiserum. We can't tell from the pattern whether the antiserum is detecting just one epitope or twenty, but we do know it is detecting *all* of them in both wells.

3) The two antigens HSA and HTf show a pattern of **non-identity** - the precipitin lines cross each other without joining at all. This indicates the two antigens are *antigenically unrelated,* they have *no* epitopes in common which are

recognized by this antiserum. This is not unexpected, since these two molecules are not related in structure or function.

4) The two antigens HSA and BSA show a pattern of **partial identity** - the lines join together, but not completely; there is a "spur" of the HSA line over the BSA line. This indicates that the two antigens are related, but not identical, with respect to this antiserum. More specifically, it means that there are at least *two epitopes* recognized by the antibody on HSA, one of which is also present on BSA. This is illustrated in the two cartoons on the right, showing HSA as bearing two epitopes ("X" and “Y”), while BSA bears only one of the two (“X”).

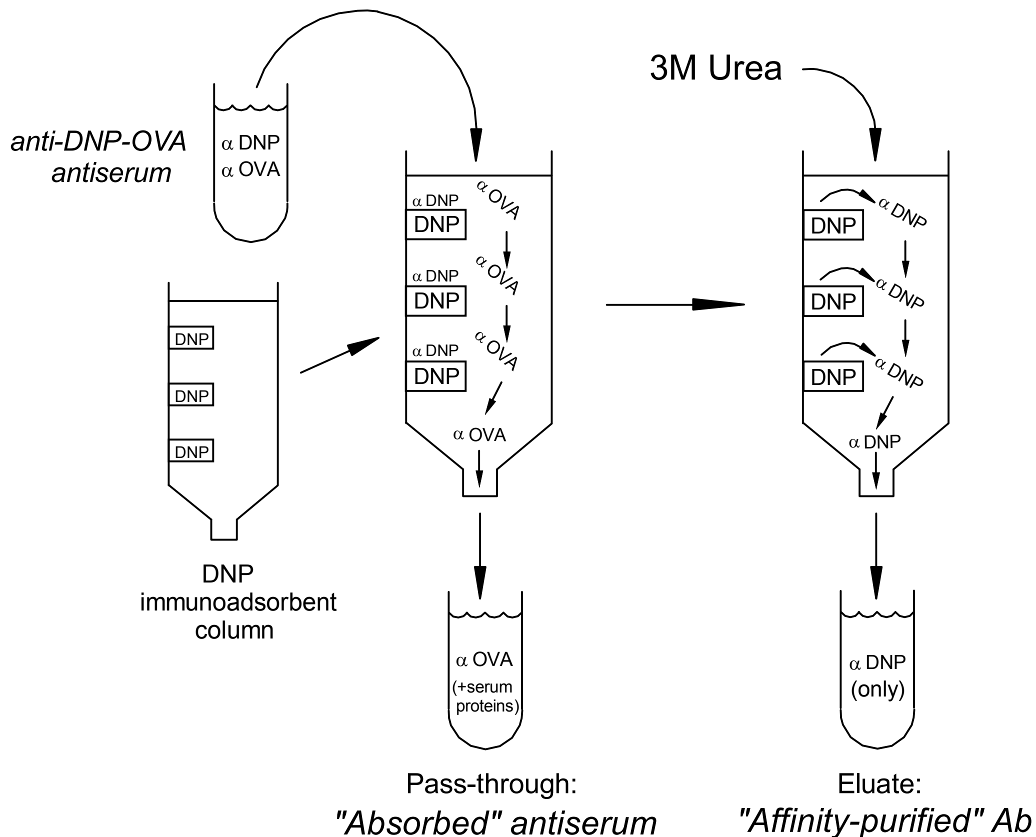
We can be more precise by stating that there are two *classes* of epitopes that the antiserum detects on HSA, and only one of the two classes is present on BSA. That is, what is shown as epitope “X” in the cartoons might actually be, say, eight separate epitopes which are all present on *both* HSA and BSA, while what is indicated as “Y” might be three epitopes present *only* on HSA.

4. ABSORPTION AND AFFINITY PURIFICATION

Affinity Purification of anti-DNP Antibody

Antigens and antibodies can be readily analyzed and purified by **affinity chromatography,** a technique widely used in chemistry. When applied to antigen-antibody systems, this procedure is often referred to as **immunoadsorption.**

Suppose we immunize a rabbit with DNP-OVA (where the protein ovalbumin is the carrier for the hapten DNP), and collect the resulting antiserum, which contains antibodies both to DNP and to the OVA protein carrier. We can separate the two kinds of antibody by preparing an immunoadsorption column as illustrated in Figure A4-1. We start by covalently coupling DNP alone, or DNP-BSA (DNP coupled to an unrelated carrier) to an insoluble matrix such as cellulose, and place it in a column. We pass our rabbit antiserum over this column; and the antibodies to DNP will bind to the DNP while everything else (including the anti-OVA antibodies) flows through. We refer to this "pass-through" fraction as an "absorbed antiserum"; the anti-DNP antibodies have been removed ("absorbed") leaving behind only anti-OVA antibodies, together with all the normal serum proteins, of course.



Affinity Purification of anti-DNP Antibody

Figure A4-1

We can then recover the anti-DNP antibodies by releasing them from the column, using either a solution of free DNP (which will compete with the bound DNP for the antibodies' combining sites), or a denaturing agent such as urea or guanidinium salts. After removing the eluting agent by dialysis, this "affinity purified" fraction contains pure anti-DNP antibodies. If, on the other hand, we use a column containing insolubilized OVA, we can recover a pure preparation of anti-OVA antibodies in the same manner.

Absorption and Affinity Purification of αHSA antiserum

This general procedure can be used to modify the specificity of antisera in more subtle ways. An antiserum prepared in a rabbit against human serum albumn (HSA), for example, will cross-react with bovine serum albumin (BSA), since the two proteins are closely related. Since distinct populations of antibodies bind to different epitopes, and binding of antibody to its antigen is reversible, we can use affinity chromatography to separate and purify the antibody populations as illustrated in Figure A4-2.

We begin with an *antiserum* generated by immunizing a rabbit with purified human serum albumin (HSA). When we analyze this antiserum "A" by Ouchterlony (bottom left) we see that there are *two distinct populations of antibody* present, one which binds to *both* HSA and BSA (bovine serum albumin), and another which binds *only* to HSA.

We then pass this antiserum over a column containing covalently bound BSA. All of those antibodies capable of binding to BSA will be retained on the column, and everything else will pass through, yielding preparation "B", which we refer to as an *"absorbed antiserum"* (more specifically, "αHSA[absBSA]". Ouchterlony analysis of this fraction (bottom center) shows the presence of a *single population of antibodies* which bind only HSA (since all those antibodies capable of binding BSA have been retained in the column).

We can now recover the antibodies bound to the column by washing it with 3M guanidium. We can remove the guanidium by dialysis and test this *"affinity purfied antibody*" fraction by Ouchterlony (bottom right). The resulting pattern tells us there is *a single population of antibodies* which bind to *both* HSA and BSA.

All three of these preparations can be useful in research and clinical settings, and it will be instructive to distinguish their properties.

● *Whole antiserum*

Whole antiserum can be used without further treatment in many assays, such as precipitation (as we have seen), agglutination, *etc*., so long as cross-reactivity between related molecules is not important. For example, a researcher might want to detect the presence of HSA in a set of samples which do not contain BSA or other cross-reactive proteins; in such a case absorption and affinity purification are unnecessary. While only a small percentage of the total protein in whole serum is specific antibody, the presence of other proteins does not interfere in most assays.

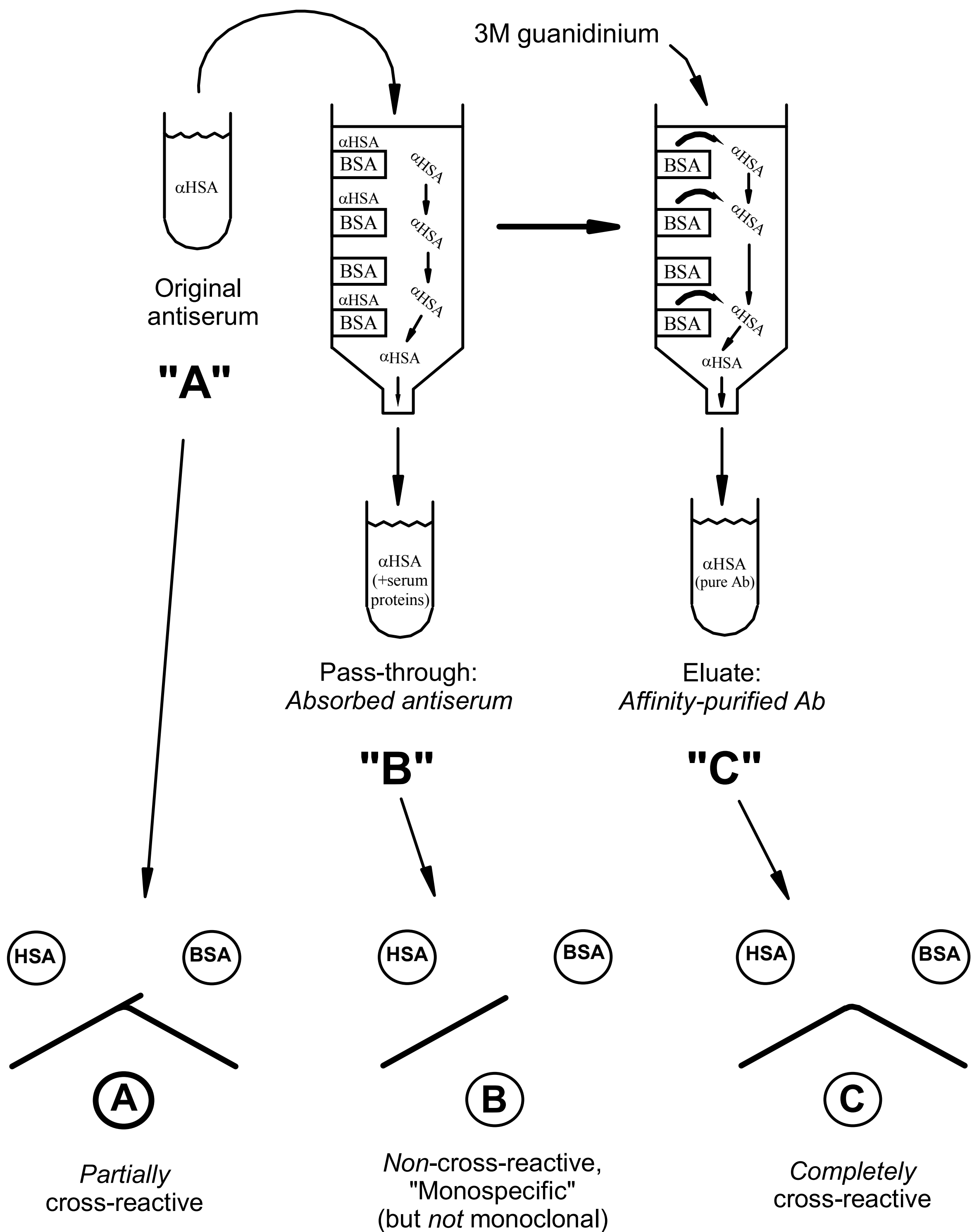


Figure A4-2

● *Absorbed antiserum*

If a forensic immunologist needs a reagent to determine if a blood stain is human or animal, then absorption of the original antiserum becomes necessary. Fraction "B" in our example will do the job in many assays (Ouchterlony, for example), showing a positive reaction only with human albumin.

● *Affinity-purified antibody*

There are a number of applications, however, for which affinity purification of antibody is either required or highly desirable. One example would be in developing an RIA or ELISA (see APPENDIX 5) to measure mammalian serum albumin, In this case we need to be able to label *only the antibody molecules* (with radioactivity or enzyme), and it is impossible to do this in the presence of the large excess of irrelevant proteins in whole or absorbed antiserum. Our *affinity-purified* fraction "C", however, contains only specific antobody molecules. This fraction could be labelled and would provide a reagent to detect mammalian serum albumin by RIA or ELISA, reacting equally well with human and bovine albumin (and presumably other related species). [QUESTION: How would you go about preparing an affinity-purified antibody for use in an RIA specific for *human* serum albumin?]

Another example would be for the therapeutic use of antibodies which are to be injected into a patient. The vast majority of irrelevant protein in either whole serum or absorbed antiserum would expose the recipient to a far greater risk of SERUM SICKNESS than affinity-purified antibody (see Chapter 5, COMPLEMENT)

5. RADIOIMMUNOASSAY - RIA

Principles of the assay

The development of the radioimmunoassay in the 1950's revolutionized the field of immunochemistry in both research and clinical applications. The RIA relies on the one property of antibody function which is a *universal feature of all antibodies*, namely the ability to bind specifically to an antigen. The degree of binding can be very accurately quantitated by making one of the components radioactive, in this example the antibody.

Our sample assay is designed to determine the concentration of insulin in some unknown samples (blood serum, urine, cell culture supernatants, etc.). We begin by coating a glass or plastic tube with a set quantity of insulin in such a way as to be permanently fixed in place. As illustrated in the top row of Figure A5-1, we then add a fixed amount of anti-insulin antibody which we have tagged with the radioactive isotope ^{125}Iodine. Most of the antibody will bind to the insulin stuck to the tube, and we then rinse the tube with saline to wash away all unbound material. We can now measure the amount of radioactivity in the tube, representing the amount of bound antibody; we determine that there are 5,000 counts per minute of radioactivity in the tube, which represents "maximum binding".

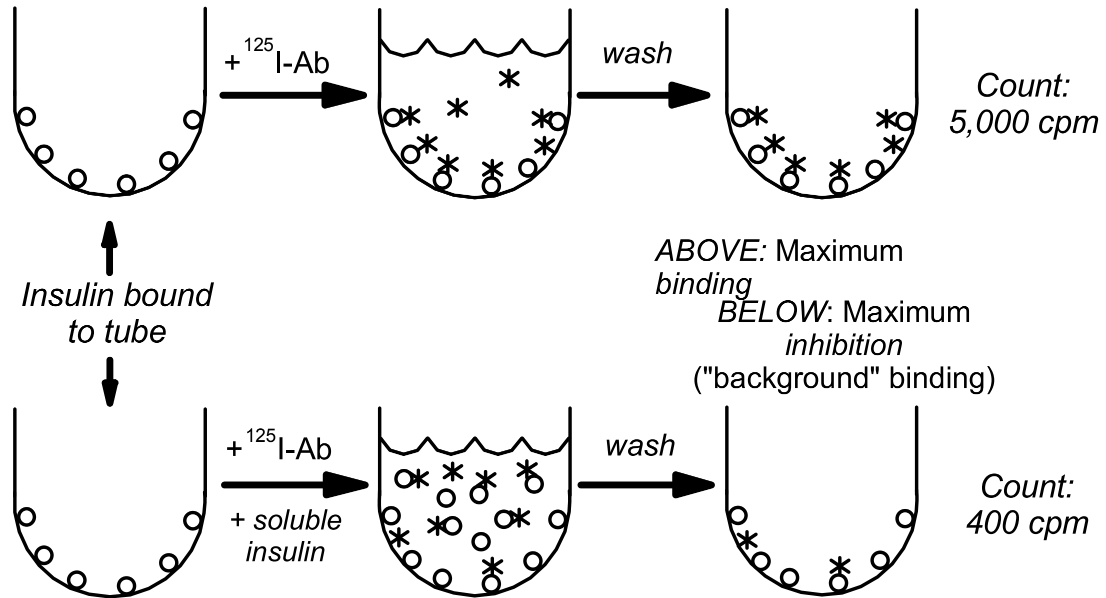
We now carry out the same exercise (shown in the second row of figures), but this time we add a large amount of *soluble* insulin ("inhibitor") together with the radioactive antibody. In this case, the antibody can bind either to the fixed or soluble insulin, and since there is more soluble than fixed insulin, most of the antibody will bind to the soluble form. This time when we rinse the tube with saline, we wash away most of the radioactive antibody together with the soluble insulin. The small amount of radioactivity which still remains behind, namely 400 counts per minute, represents some antibody (or contaminating radioactive material) sticking nonspecifically to the assay tube, or "background binding".

This defines the principles of the RIA – *specific binding of antibody to antigen can be sensitively measured, and this binding can be inhibited by the addition of soluble antigen*. A small amount of soluble antigen will inhibit binding only slightly, a large amount will inhibit more, and we can use this relationship to determine the concentration of inhibitor in any unknown sample..

Inhibition curves

If we have a series of experimental tubes to which we add increasing amounts of inhibitor, we can generate inhibition curves such as those shown at the bottom of Figure A5-1. One common practice is to make serial dilutions of the inhibitor from an original stock (say 1:2, 1:4, 1:8, etc.) and add a fixed volume of each dilution to each assay tube. There are a variety of methods for plotting such data, and the one we will use is shown at the right, plotting increasing inhibition (from 0 to 100%) on the Y-axis, *versus* increasing dilution of inhibitor on the X-axis. Using our standard volume of a *low dilution* (*i.e.* a *large amount*) of inhibitor, we see 100% inhibition. As we increase the dilution (*i.e.* decrease the amount) of the inhibitor, we see less and less inhibition, until the curve finally flattens out at 0% inhibition. In this example we see 50% inhibition at a dilution of about 1:40, and we refer to this curve as exhibiting a "titer" of 1:40

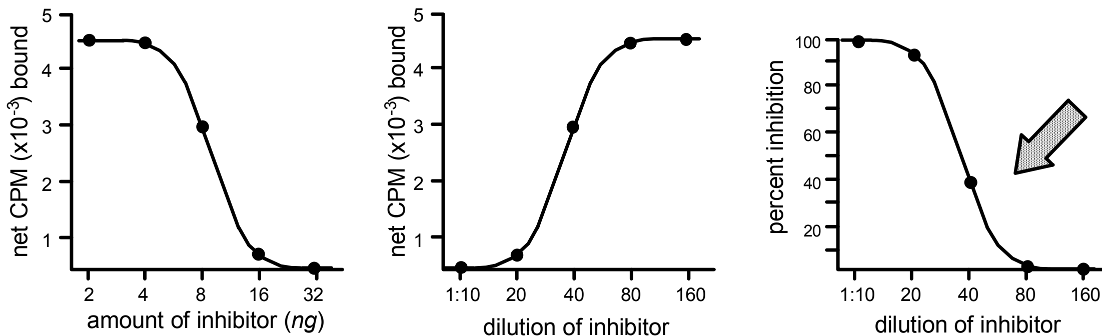
RadioImmunoAssay (RIA)



ASSAY: Fixed quantity of *bound* Ag (insulin= o)
Fixed quantity of ^{125}I-Ab (= ∗)
Variable quantity of *soluble* inhibitor (insulin= o)

Maximum total binding = 5000 cpm.
Maximum inhibition yields 400 cpm bound (=100% inhibition).
Maximum specific binding = 4600 cpm (=0% inhibition).

VARIOUS WAYS TO PLOT RESULTS...



Note: Display and interpretation of data are identical for RIA and the more commonly used ELISA

Figure A5-1

How do we use this assay to determing the concentration of insulin in an unknown sample? Consider the inhibition curves shown below in Figure A5-2, where our standard insulin solution shows a titer of 1:40, while the unknown sample has a titer of 1:160. We conclude that the unknown sample (in its original, undiluted form) contains insulin at a concentration four times greater than our standard. If our standard insulin stock has a concentration of 20 pmol/L (picomoles per liter), which we can determine by looking at the manufacturer's label, then the unknown sample must be at 80 pmol/L.

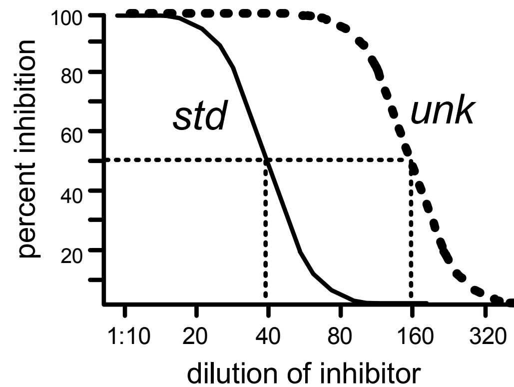


Figure A5-2

ENZYME-LINKED IMMUNOADSORBENT ASSAY (ELISA) versus RIA

As mentioned in APPENDIX 2, RIA's have been superceded in many of their applications by Enzyme-Linked Immunosorbent Assays (ELISA), which can be as sensitive than RIA's while avoiding the hazards and expense associated with the use of radioactive materials. We have introduced this class of binding assays using the RIA as the primary example because it is conceptually simpler, but the principles of the ELISA are the same as those described above, and the data are plotted and interpreted in precisely the same manner as RIA's. In fact, there is no way to know simply by examining an inhibition curve whether it was generated in an ELISA or RIA.

6. ANALYSIS OF THE ANTIBODY COMBINING SITE BY EQUILIBRIUM DIALYSIS

Equilibirum dialysis was the first technique used to determine the thermodynamic properties of antigen-antibody binding. It revealed that this reaction was a **non-covalent and reversible reaction**, that the basic IgG-like unit had exactly **two combining sites**, and it determined the values of the equilibrium constant (**affinity**) of the binding reaction.

Requirements for the Analysis

1) We must have a *pure preparation of antibody,* not just a gamma-globulin preparation or an ammonium sulfate fraction; the reason for this is that we must know the *molar concentration* of antibody. We must, therefore, also know the molecular weight of IgG antibody, which is about 150,000.

2) We must carry out the analysis using a *monovalent, low molecular weight antigen* such as a hapten. The reasons for this are twofold: first, the hapten must be *small* enough to pass across a dialysis membrane (the antibody, of course, is too large); and second, it must be *monovalent* to fulfill the assumptions we will make regarding independent binding to multiple antibody combining sites on the antibody.

Dialysis Apparatus

The apparatus consists of two small chambers connected only through a semipermeable membrane, as diagrammed below. In one chamber we place a known amount of total antibody (Ab_T), in the other a known amount of total hapten (H_T); the starting condition is shown on the left of the figure below.

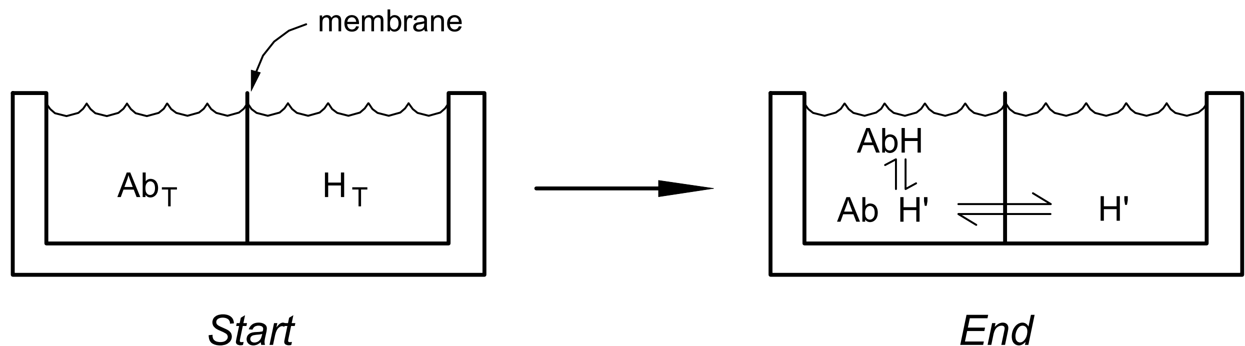


Figure A6-1

The two chambers are then allowed to reach thermodynamic equilibrium, which may take one or two days. During that time, the hapten will diffuse across the membrane, and some of it will be bound by the antibody in the other chamber, as indicated on the right side of the diagram.

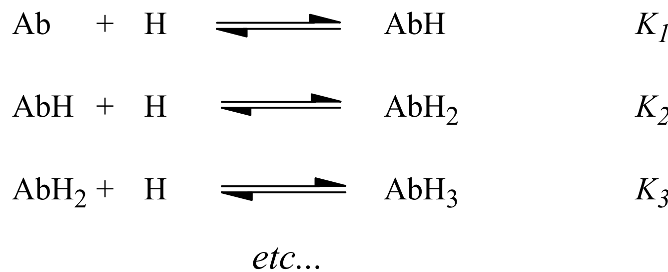
The key point is that the concentration of *free* hapten on both sides of the membrane will be the same at equilibrium, indicated as H'. In addition, there will be *free* antibody (Ab) and antibody bound to hapten (AbH).

Since H_T = 2H' + AbH, we can determine the value of AbH (H_T is known, and H' is measured). Then, since Ab_T = Ab + AbH, we can also determine Ab, or the concentration of *free* antibody.

Analysis

The experimental data are collected by measuring the concentration of free hapten at equilibrium, having started with a series of different initial hapten concentrations. The basic rationale is that *at any given hapten concentration, a higher affinity antibody will bind a higher proportion of hapten.*

We can rewrite the reaction as a *series* of reactions, as follows:



$$Ab + H \rightleftharpoons AbH \qquad K_1$$

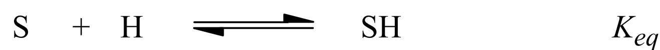
$$AbH + H \rightleftharpoons AbH_2 \qquad K_2$$

$$AbH_2 + H \rightleftharpoons AbH_3 \qquad K_3$$

etc...

Each reaction represents the binding of one additional hapten molecule to an antibody molecule, up to some unknown maximum (which depends on the total number of sites actually present on each antibody molecule); each reaction is governed by an equilibrium constant, K_1, K_2, etc.

> ***Assumptions:*** *(1) All binding sites on a single Ab molecule have the same affinity, and (2) binding of H by different combining sites on the same antibody molecule is independent; i.e.* binding of H to one site does not help or hinder binding of H to another. If these assumptions are correct, then all the reactions above can be represented by a *single* reaction with a *single* equilibrium constant, where "S" represents a single antibody-combining site.



$$S + H \rightleftharpoons SH \qquad K_{eq}$$

By the definition of the equilibrium constant:

$$K_{eq} = \frac{[SH]}{[S][H]}$$

This can be shown to be equivalent to the following equation:

$$\frac{r}{c} = Kn - Kr$$

where:

r	=	moles H bound per mole of antibody
c	=	concentration of free H, or [H]
K	=	K_{eq}, defined above
n	=	number of binding sites per Ab molecule (or [S] / [Ab])

This equation is that of a straight line of the form Y = b + mX, where Y is replaced with R/c, the constant *b* is replaced with Kn, and mX is replaced with -Kr. If we plot our data, we *expect* to get the straight line labeled "*theoretical*", as shown below:

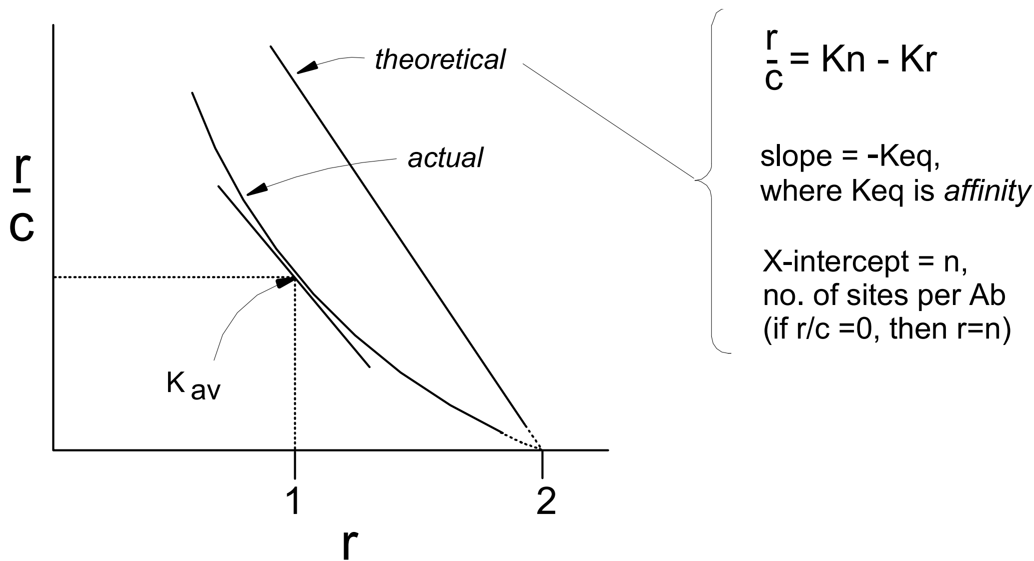


Figure A6-2

Two important values can be determined from this straight line; first, *the slope (*actually the *negative* slope*) gives us Keq*, the equilibrium constant, which we have defined as the *affinity*; second, *the X-intercept gives us the value of n,* the number of combining sites per antibody molecule. In this graph, the theoretical line is drawn so that it crosses the X-axis at a value of 2.

So far we have been dealing with a theoretical straight line; but when we actually carry out such an analysis with the rabbit anti-DNP antibodies and plot the results, we get *not a straight line, but a curve* such as shown in the figure above (labeled "actual").

- *The curvature indicates that the antibody preparation is heterogeneous with respect to affinity.* There are antibodies with high and low affinities, and everything in between. (The degree of curvature can, in fact, be used as a measure of the degree of antibody heterogeneity.)

- *If we extrapolate the curve to the X-axis, we can estimate a value for n of 2;* this means that there are *two* combining sites for DNP on each IgG antibody molecule.

- *If we carry out this analysis with homogeneous IgG anti-DNP antibodies* (for instance with a human DNP-binding myeloma, or an anti-DNP hybridoma), we *do* get a straight line with an X-intercept of 2, and a slope which defines the affinity of that particular antibody (as shown by the "theoretical" line in Figure A5-2).

The shape of the curve can be understood intuitively. At very high values for r/c, that is, at very low levels of *free* hapten, those antibodies with high affinities will more effectively compete for what little hapten is present; therefore, binding by the high affinity antibodies predominates and the slope of the curve is *high* (the curve is steep) in that region. At *low* values of r/c there is plenty of hapten to go around, and the lower affinity antibodies can contribute a larger proportion of the binding; the curve is shallower in this region, representing a lower overall affinity.

One can define the "average affinity" (K_{av}) of a heterogeneous antibody population as the slope of the curve where r =1, that is, at the point where half of the available sites are occupied; this is indicated as K_{av} in Figure A6-2.

To better understand the relationship between these experimental data and the "real-life" functions of antibodies, let's define two terms which are distinct, but easily confused, namely AFFINITY and AVIDITY.

AFFINITY: *The strength of binding of a single antibody combining site with its epitope; the equilibrium constant of the binding reaction*. Conventional antibodies have measurable affinity constants in the range of 10^4 to 10^{10}; higher and lower values certainly exist in nature, but are generally difficult to measure accurately. *Many methods* other than equilibrium dialysis exist for determining affinity constants, but they are all measures of the *interaction of a single combining site with its antigen*.

AVIDITY: *The strength of binding of a multivalent antibody to a multivalent antigen; a measure of the ability of antibodies to form stable complexes with their antigens.* The *avidity* of an antibody depends not only on the *affinity,* but also on the *valency* of both antibody and antigen, and on various physical properties of both. Avidity does not have a standard thermodynamic definition, although it can be defined in a relative and *ad hoc* manner within a specific given experimental context.

The difference between affinity and avidity may be illustrated by the following diagram, showing the two-step binding of a bivalent antibody to a multivalent antigen molecule:

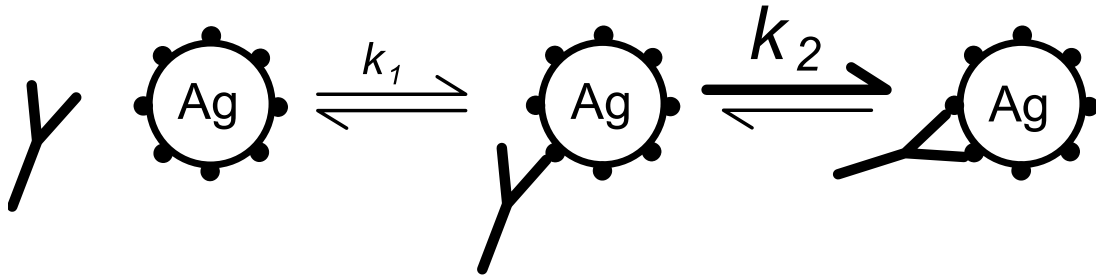


Figure A6-3

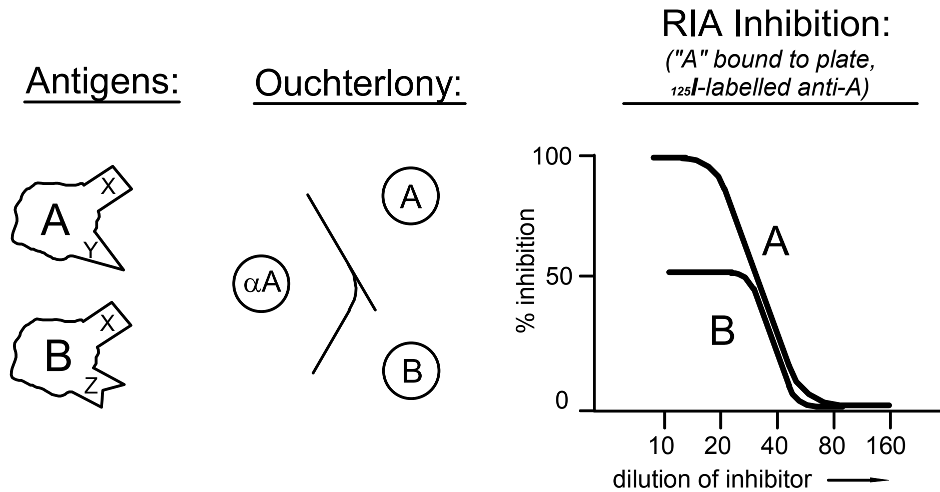
The first step of binding occurs at a *rate* (shown as k_1 above) determined by the affinity of the combining site for its epitope (*note that we are now discussing reaction rates, not equilibrium constants*). The second step, however, occurs with a much higher rate constant, k_2; once the first site has bound to the antigen, the likelihood of the second site binding is much higher, since the second site is held in close proximity to another epitope. This *cooperativity* between binding sites on an antibody molecule makes the *effective binding to antigen much stronger than would be predicted simply by the equilibrium constant*; **avidity** is the term used to describe this greater strength of binding.

Antigens in nature are often polyvalent and highly repetitive structures. The strength of binding by antibodies to such antigens (microbial cell wall components, for instance) is much higher than the antibodies' affinities would predict, because of the effect of *cooperative binding*. This effect becomes even more important in the case of polymeric IgA, and of IgM which can have up to 10 combining sites in a single antibody molecule (although all of the sites may not necessarily be capable of binding antigen simultaneously).

7. CROSS-REACTIVITY

We've seen examples of serological cross-reactivity illustrated by Ouchterlony precipitation patterns. Adding the use of RIA allows us to distinguish two different modes of cross-reactivity.

Figure A7-1 shows the kind of cross-reactivity we have already seen, due to the presence of multiple distinct epitopes which may be unique or shared between antigens. At the left we see the structural basis for such cross-reactivity - antigen "A" bears two epitopes labeled "X' and "Y", antigen "B" also has epitope "X", together with a different epitope "Z". Using an antiserum prepared against antigen "A" shows the Ouchterlony pattern we recognize as *partial identity* - a shared line of precipitation due to the presence of epitope "X" on both antigens, but with a "spur" of "A" over "B" because epitope "Y" is present only on antigen "A".

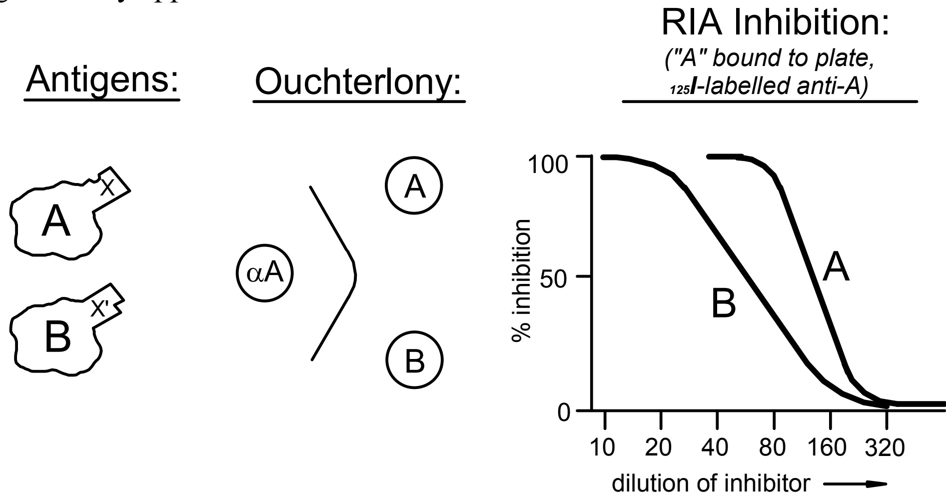


Cross-Reaction due to Multiple Distinct Epitopes

Figure A7-1

If we carry out an analysis by inhibition of RIA (at the right), we also see evidence of partial cross-reactivity. The assay consists of antigen "A" bound to the tube incubated with affinity-purified radiolabeled antibody to the same antigen. Thus we have labeled antibodies to both epitopes "X" and "Y". If we inhibit with a sufficient amount of purified antigen "A", we can inhibit 100% of the antibody binding, as expected. However, when we use antigen "B" as inhibitor, we find that no matter how much we add we can only inhibit 50% of the binding. This is because some of the anti-A antibody (50% in this example) is directed against epitope "Y". Since antigen "B" doesn't have this epitope, it cannot inhibit this binding. (Note that the putative presence of epitope "Z" on antigen "B" is irrelevant in our example, since there are no antibodies to this epitope in the system.)

Let's consider another situation, where two antigens each have one epitope which is related, but not quite identical, to that on the other (indicated as X and X' below). In this case the pattern we see in Ouchterlony is one of *complete* identity, despite the fact that we expect antibodies to epitope X to bind less strongly to X'. But as long as the antibodies bind X' *sufficiently strongly* to cause precipitation, Ouchterlony analysis will not distinguish the two antigens - they appear identical.



Cross-Reaction due to Related Epitopes

Figure A7-2

When we analyze these antigens by RIA, however, we see something different. Antigen B can, indeed, completely inhibit the binding of anti-X to epitope X, but since its own epitope X' is recognized by the antibody with lower affinity (therefore resulting in lower avidity), it has a more difficult job inhibiting. This is reflected in the inhibition curve for antigen "B" which shows a shallower slope than that for "A", and a greater amount of antigen necessary to finally achieve complete inhibition.

Recognizing this property of cross-reactive antigens is of practical importance in interpreting RIA results. One explicit assumption in using RIA to determing the concentration of an antigen (as, for example, insulin in APPENDIX 5), is that the antigen in the unknown samples is *identical* to that in the standard. If we see an inhibition curve which is *not* identical in shape to that of the standard, then we know that the material in the unknown sample is structurally different from that in the standard, and we cannot draw any conclusion regarding relative concentrations.

8. **COMPLEMENT FIXATION ASSAY** (see Chapter 5, COMPLEMENT)

Many different methods exist to measure the levels and degree of activation of the various complement components, most of which are used only in highly specialized labs devoted to complement research. The classical *Complement Fixation (CF)* assay, however, has long been widely used by biologists of all kinds as a simple and sensitive way to detect and quantitate a wide variety of antigens and antibodies. While it is still sometimes used in both research and clinical situation, many of its applications have been replaced by ELISA or other more sensitive and quantifiable methods.

Let's illustrate the principles by constructing a complement-fixation assay to detect a hypothetical viral antigen (VA) in several unknown serum samples.

> We begin by making "sensitized" erythrocytes, which are simply RBC coated with anti-red blood cell antibodies; we call these EA (*E*rythrocyte + *A*ntibody). In the absence of complement these cells remain intact, but they will be lysed if exposed to any source of complement; these EAs are our ***indicator cells.***
>
> Next, we prepare a standard source of ***complement***, usually fresh guinea pig serum diluted appropriately. The dilution is such that it contains just enough complement to completely lyse a standard amount of EA.
>
> We also prepare a ***standardized antibody*** to the antigen, by immunizing a rabbit with purified VA. Before use the rabbit antiserum is heat-treated (56° C for 30 min) to remove its inherent complement activity.
>
> We then take a series of test samples (for example, heat-treated serum samples from various patients), mix them with a standard amount of our antibody, and incubate the mixture for an hour (allowing the antibodies to react with the antigen, if it is present). To this mixture we then add a standard amount of the guinea pig serum as a source of complement, and after a short time (to allow the AgAb complexes to interact with the complement) we add a standard amount of the sensitized erythrocytes (EA). We incubate the samples at 37° C for a half hour and examine them visually (or spectrophotometrically) for lysis; a suspension of intact RBC is cloudy, but it becomes transparent when the cells are lysed.

The protocol would be as shown on the table below (where "*unk*" refers to an "unknown" or test sample).

Tube 1 tells us that the erythrocytes have been properly sensitized and that our guinea pig serum (as a source of complement) is effective. Tube 2 shows us that the antibody alone does not interfere with the complement-dependent lysis of the sensitized RBC. In tube 3, the antibody will form complexes with the added VA protein, and these complexes will consume ("fix") the complement; therefore, there will not be enough complement left to subsequently lyse the indicator cells. Repeating this test with two unknown samples in tubes 4 and 5 tell us that sample No. 2 must contain the viral antigen VA, while sample No. 1 does not. We carry out one additional negative control, by re-testing *unk* sample number 2 in the *absence* of specific antibody. As expected (tube #6), no complement is fixed, and the indicator cells are lysed.

Tube No.	Sample					Result	Interpretation
1)	(saline alone	+	C)	+	EA	lysis	neg control
2)	(Ab + saline	+	C)	+	EA	lysis	neg control
3)	(Ab + VA	+	C)	+	EA	no lysis	pos control
4)	(Ab + *unk* No. 1	+	C)	+	EA	lysis	no VA present
5)	(Ab + *unk* No. 2	+	C)	+	EA	no lysis	VA present
6)	(*unk* No. 2	+	C)	+	EA	lysis	neg control, OK
7)	(Ab + *unk* No. 3	+	C)	+	EA	no lysis	VA present??
8)	(*unk* No. 3	+	C)	+	EA	no lysis	anti-complement activity

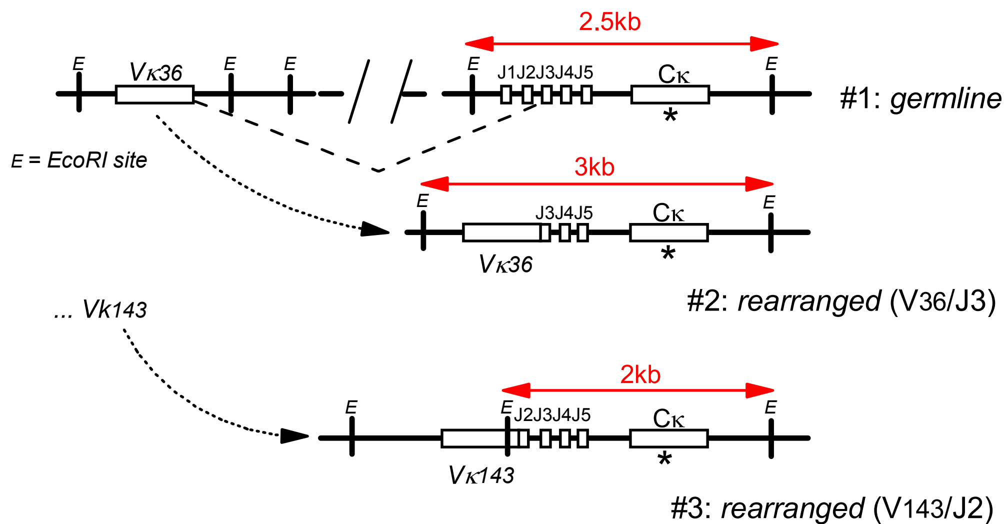
However, the last two tubes (7 and 8) illustrated a possible complication in interpreting these results. Tube 7 shows no lysis, suggesting the presence of viral antigen in sample #3 (as was the case with sample #2). However, when we run this same sample again *in the absence of specific Ab,* there is still no lysis, although we expect the same result as in our negative controls (tubes 2 and 6). This shows that serum #3 inhibits complement activity by itself, a property referred to as "anti-complementary;" we therefore can say nothing about the presence or absence of viral antigen in this sample unless we find a way to remove this activity. Knowing of this possibility, it is clear that we must repeat this control test for *every* positive sample (as we did for sample #2 in tube 6) to confirm that the positive result is not the result of anti-complementary activity. (Such activity might be due to the presence of unrelated immune complexes in the serum, the presence of antibodies which happen to recognize components of the guinea pig complement, or the presence of drugs which inhibit complement.)

Despite these cautions, complement fixation assays are still used in a variety of specialized applications. They can provide very sensitive tests for any *antigen* to which an antibody is available, or, by changing the variables, can be used to test for the presence of *antibody* to a standard antigen. They can also be used to *quantitate* the level of antigen (or antibody) by carrying out the tests with varying dilutions of the unknown, and accurately determining the level of lysis of the indicator cells by colorimetric means.

9. ANALYSIS OF KAPPA-CHAIN GENE REARRANGEMENTS BY SOUTHERN BLOTTING

Determining the nature of gene rearrangements in tumor cell populations can be a powerful tool to aid in diagnosis of a variety of cancers. Let's examine how the Southern blotting technique can be applied to examining kappa-chain rearrangements in cells of the B-cell lineage.

At the top of Figure A9-1 (#1, germline) is a schematic representation of the genomic configuration of the human kappa-chain gene complex, showing the constant region with its five associated J-segments at the right, and one of the many V-regions at the left. Also indicated are the positions of "EcoRI" sites, short DNA sequences which are specifically cleaved by this restriction enzyme. If this *un-rearranged* configuration of DNA were treated with EcoRI, the only fragment containing Cκ sequences would be the 2.5kb fragment indicated.



Germline and Two Rearranged Configurations of Human Ck

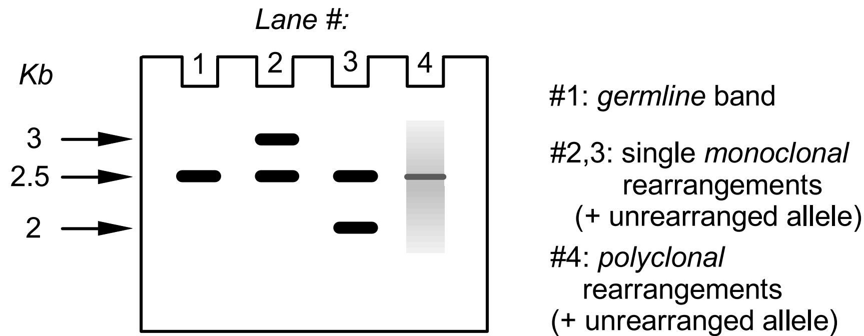
Figure A9-1

If we look at the configuration of this region of DNA in B-cells which have rearranged their κ-chain complex, we might see any one of a large number of different results, two of which are illustrated in the figure. In the first case (line #2), the rearrangement has removed the DNA containing the EcoRI site just to the left (3' side) of J1, so that the the Cκ region now happens to be on an EcoRI fragment 3kb in length. In the second case (line #3), the Vk gene segment that was used happens to have an EcoRI site near it's 5' end, so the Cκ gene is now on a fragment 2kb long. The hundreds of different possible Vk/Jk rearrangements will yield many different results; in unrearranged DNA the Ck gene segment will exist *only* on a 2.5kb EcoRI fragment, in rearranged DNA it could end up on *any* sized fragment.

How can we use this information to analyze cell populations and help diagnose B-cell tumors? Let's use the Southern blotting technique to examine DNA from various sources. We extract DNA from a cell population, treat it with EcoRI, then separate the resulting fragments according to size by electrophoresis in an agarose gel. We then transfer the separated fragments from the gel onto a sheet of nitrocellulose, incubate the sheet with a

radioactive DNA probe which will hybridize with its complementary sequences, and we can determine the location of the radioactivity by autoradiography.

A schematic showing the results of such an experiment is shown in Figure A9-2. Various samples of EcoRI-treated have been separated in an electric field from top to bottom, the smaller fragments moving the farthest. The size of fragments at each position is indicated on the left.



Southern Blot Hybridized with Cκ Probe

Figure A9-2

Sample #1 consists of DNA from a cell population which has *not* rearranged Ck, for example skin, liver, kidney, brain, or any source *other* than cells of the B-cell lineage. Since no rearrangement has taken place, the only place we see Cκ-hybridizing sequences is at the position of the germ-line 2.5kb fragment.

Samples #2 and #3 have been prepared from two different plasmacytomas, Ig-producing human tumors. Since cancers are monoclonal in their origin, all the plasma cells in a particular tumor have undergone exactly the same rearrangement(s). In this case, each tumor has rearranged *one* of its two copies of Cκ, but *not the other*, resulting in the appearance of two bands in each lane, the familiar 2.5kb germ-line band (non-rearranged) and a single, unique new band (rearranged).

Sample #4 is what we would expect with DNA prepared from a population of normal B-cells, taken (for example) from spleen, lymph node or blood. Each B-cell in the population has rearranged at least one Cκ gene, some have rearranged both. But each cell has rearranged in a unique fashion, resulting in a heterogeneous mix of many different fragments which contain the Cκ segment; this is seen as the "smear" of radioactivity in the right-hand lane. Since some of the B-cells have rearranged only one of their two alleles, all the non-rearranged alleles contribute to the visible band at the germ-line position.

We can apply this approach, for example, to help distinguish a B-cell tumor from more benign forms of lymphoid hyperplasia in a particular patient. In the case of a tumor, Southern blotting will show a pattern indicating monoclonal rearrangement (one or two discrete non-germline bands as in Lanes #2 and #3 above), whereas a pattern of polyclonal rearrangement (as in Lane #4) would indicate non-cancerous form of B-cell hyperplasia. The same approach can be used to help in the diagnosis of T-cell tumors, in that case using probes specific for T-cell receptor sequences rather than immunoglobulins.

10. GENETICS OF INBREEDING

"...the development of inbred strains has constituted probably the greatest advance in all cancer research." (W.E. Heston, 1963)

"The introduction of inbred strains into biology is probably comparable in importance with that of the analytical balance into chemistry." (Hans Grüneberg, 1952)

IMPORTANCE OF INBRED STRAINS OF MICE

One of the major obstacles in biological research of all kinds is the *genetic variation* inherent in most natural populations of organisms. Individual humans as well as experimental animals vary widely in their physiological properties and biological responses, and much of this variation (although not all of it) is genetically determined. One particular manifestation of such variability was recognized by cancer biologists more than a century ago, namely the inability to reliably transfer growing tumors from one mouse in a laboratory colony to another. It was also recognized that occasional successful transplants generally involved closely related animals, and depended on their degree of genetic relatedness; this is the basis for the special relevance of inbreeding to cancer research alluded to by Heston, above.

Shortly after the turn of the twentieth century, the geneticist C.C. Little developed the earliest inbred strains of mice, which for the first time provided *genetically uniform* mammals for research. The importance of such animals cannot be overstated, as attested to by the two quotations above. While their development was originally stimulated by their need in tumor transplantation, inbred mice have provided essential tools for research in genetics, physiology and behavior, as well as in all aspects of immunology. They have provided animal models for the study of many human diseases, including amyloidosis, polyuria, diabetes, muscular dystrophy and many others. In an immunological context, the use of inbred strains has been indispensible for the success of *adoptive transfer systems*, of which we have discussed several examples, as well as for the elucidation of the biological roles and chemical structure of products of the *Major Histocompatibility Complex* (MHC).

PRODUCTION OF INBRED MICE: BROTHER-SISTER INBREEDING

We can illustrate the basic features of the process of inbreeding, and of the resulting inbred strains, by the protocol originally used by Little. Let’s begin with two randomly chosen mice taken either from the wild, or, as was more commonly done, from the colonies of mouse "fanciers" who kept them for amusement or for sale. The two mice we start with may or may not be related, but in either case will differ in the allelic forms of genes they carry at many genetic loci.

Let’s consider a *randomly* chosen genetic locus which has two allelic forms, which we name **A** and **a**. *NOTE*: We do not have to know what this locus is, nor do we need to be able to distinguish its genotypes in order to understand the analysis that follows.

Suppose we happen to start with two individuals *heterozygous* at this locus; the resulting cross can then be diagrammed as follows:

Parents: **Aa** X **Aa**

Offspring (F_1) **AA** **Aa** **aA** **aa**

Frequencies:

AA: 25%
Aa: 50%
aa: 25%

Using simple Mendelian genetics, we can see that there are three possible genotypes for the offspring of such a cross, and their frequencies are given above. If we then *randomly* choose two of these offspring to mate for the next (F_2) generation, there are six possible crosses which may result. These six possibilities, together with the probability of having chosen each, are shown below:

			probability of cross		*next generation*
AA	X	**AA**	1/16	⟶	offspring *all* **AA**, p=1/16
AA	X	**Aa**	1/4	}	
AA	X	**aa**	1/8	}	offspring varied
Aa	X	**Aa**	1/4	}	
Aa	X	**aa**	1/4	}	
aa	X	**aa**	1/16	⟶	offspring *all* **aa**, p=1/16

For example, the probability of our having randomly chosen two animals of genotype AA is simply 1/4 X 1/4, or 1/16 (= 6.25%). The probability of the second combination (**AA** X **Aa**) is 1/4 X 1/2 X 2 = 1/4 (we multiply by 2 to take into account the fact that there are *two* possible choices which will yield this cross, namely **AA** X **Aa**, and **Aa** X **AA**; the same situtation holds for all crosses in which the two individuals differ from one another).

The first and last possibilities in this list (**AA** X **AA**, and **aa** X **aa**) are special cases: for both of these crosses *all offspring will be identical to their parents*. If we add the probabilities for these two cases, we find that there is a 12.5% likelihood (1/16+1/16= 1/8) that we will *happen* to have picked a pair of identically homozygous animals in this generation. ***If this happens, regardless of which of the two possibilities we have chosen, all subsequent generations will be homozygous at this locus, as long as we keep to the brother-sister mating scheme.***

This is equivalent to stating that *12.5% of all genetic loci will be homozygous* in this generation, based simply on chance. Adhering to the brother-sister mating scheme ensures that once we happen to have chosen two animals homozygous for a given locus, *no new heterozygosity can ever be introduced*. Thus, as we continue this mating scheme, generation after generation, the resulting animals progressively approach a condition of *uniform homozygosity at all loci*. It is important to recognize that this analysis does not depend on our knowing anything about any of the genetic loci involved, nor does it rely on any deliberate selection on our part.

After 20 generations of such inbreeding, the overall homozygosity is about 98.5% (the "*coefficient of inbreeding"* = 98.5%), and we are entitled to call this line an *inbred strain*. Brother-sister inbreeding is continued throughout the maintenance of all inbred strains, and the overall level of homozygosity continues to increase, until that point at which it is counterbalanced by the appearance of spontaneous mutations (at a very low rate).

The oldest inbred strains of mice and rats date back to about 1909, and many have gone through hundreds of generations of brother-sister mating since then. Commonly used strains of mice include C57Bl/6, C3H, BALB/c, DBA/2 and AKR. There exist well over 200 strains (and many substrains) of inbred mice and scores of inbred rat strains, as well as inbred strains of rabbits, chickens, hamsters and guinea pigs.

In addition to the *random* brother-sister mating scheme described above, *additional selection can be imposed* during the early stages of inbreeding for the establishment of desired traits. This has resulted in the production of inbred strains of mice and rats with high (or low) incidence of various tumors, dental caries, blood pressure, and many other physiological or behavioral characteristics.

PROPERTIES OF INBRED STRAINS

Inbred strains have several properties which distinguish them from conventional laboratory or wild animals.

1) All members of a particular strain are *genetically uniform* (or *"isogenic*"); they are as identical to each other as human identical twins.
2) Furthermore, they are *homozygous at all loci* (*unlike* human twins).
3) Inbred strains "*breed true"*; that is, if two members of an inbred strain are mated, their offspring are also members of that strain; they are uniformly homozygous and identical to all other individuals of that strain.

Individuals of inbred strains are often (although not always) fairly delicate and of limited fertility, which tends to make them difficult to maintain and expensive to use.

PROPERTIES OF F_1 INBRED CROSSES

Individuals of two different strains are often mated to produce "F_1 crosses". For example, a female C57Bl/6 mouse might be mated with a male of strain DBA/2, yielding an F_1 cross named (C57Bl/6xDBA/2)F_1, or, more briefly, "B6D2F1".

Such F_1 crosses between inbred strains posess important and useful properties:

1) Like inbred strains, they are also *genetically uniform* (*"isogenic*"); they are homozygous at all those genetic loci for which the parents happen to be identical.
2) However, they are *not homozygous* at all loci, unlike their inbred parents - in this respect they are more similar to human identical twins than are their inbred parents. In fact, they are heterozygous at all those loci (but *only* at those loci) for which the two parental strains carry different alleles.

3) The property of being an F_1 hybrid *does not breed true*; a cross between two B6D2F1 mice does *not* yield another B6D2F1 mouse, but a mixture of genetically heterogenous offspring. A regular supply of F_1 hybrids therefore requires the continuous maintenance of *both* inbred parental strains.

4) The products of F_1 crosses between inbred strains are often very *hardy* and of higher fertility, exhibiting what is called "hybrid vigor". This generally makes them easier and less expensive to use, and such animals are widely used for many experiments which require genetic uniformity, but not homozygosity.

11. MIXED LYMPHOCYTE REACTION (MLR)

Allelic differences in the MHC complexes between two individuals can be detected by transplant rejection, and by specific antibodies to the transplantation antigens. They can also be detected by the MLR.

If lymphoid cells (spleen cells, for instance) from two different strains of mouse are mixed and allowed to incubate in culture for a few days, each population will be triggered by the foreign antigens of the other. One result of this triggering is a proliferative response which can be detected by increased uptake of tritiated thymidine.

Cells	Thymidine uptake, CPM
BALB/c + BALB/c	800
BALB/c + C57Bl	28,400

This is referred to as a ***"two-way" MLR***, since both BALB/c and C57B1 cells can respond to the MHC antigens of the other. One can make this test more informative by turning it into a ***"one-way" MLR*** . This is done by treating one of the two cell populations with the drug Mitomycin C, or with a high dose of X-irradiation. In either case, the ability of the cell population to proliferate is eliminated, but its ability to act as a stimulator is not affected.

Cells (*=mitomycin treated)	Thymidine uptake, CPM
BALB/c + BALB/c*	800
BALB/c + C57Bl	27,200
BALB/c*+ C57Bl	21,700
BALB/c*+ C57Bl*	950
BALB/c + (C57BlxBALB/c)F_1	21,500
BALB/c + (C57BlxBALB/c)F_1*	22,900
BALB/c*+ (C57BlxBALB/c)F_1	1,100

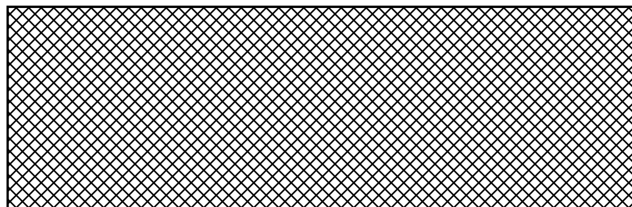
Mitomycin treatment of *either* cell population in the first case reduces the response (since fewer cells are proliferating), while treating *both* eliminates the response altogether. In the second case, we are mixing BALB/c cells with F1 cells; this is *already* a one-way response, since the standard rules apply, and the F1 cannot respond to the parental cells (the F1 cells see nothing "foreign" on the parent). In this case, mitomycin-treating the F1 has no effect, while treating the BALB/c parent cells eliminates the response.

The MLR differs from the transplantation reaction in one major way, however, in that the *antigens responsible for MLR are primarily Class II antigens of the MHC.* Mixing cells from mice differing only at K or D will yield weak MLR reactions, while cells differing only in the I-region will give strong reactions. It is for this reason that the MLR has been of considerable importance in tissue typing for human transplantion. Antibodies against Class II antigens have generally been difficult to produce, and MLR has allowed transplants to be matched for Class II antigens as well as Class I antigens (which are readily typed serologically). It is for this reason that Class II antigens have generally been referred to as LD ("lymphocyte determined"). antigens, as apposed to the Class I, SD ("serologically determined") antigens (see Figure 11-5).

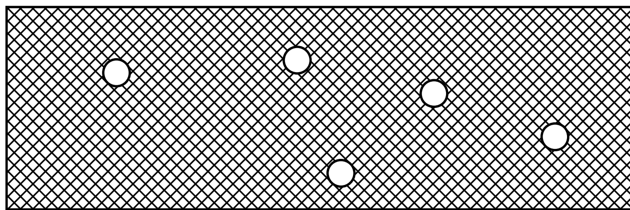
12. PLAQUE-FORMING CELL (PFC) ASSAY

The development in the 1960's of the PFC (plaque-forming cell) assay by Jerne represented a major advance in the progress of cellular immunology. It provided, for the first time, a simple and reliable method for determining the *number of individual antibody-forming cells* (AFCs) in a cell population. The kinetics of antibody-forming cell production could then be studied without having to rely solely on the levels of serum antibody, which is related only very indirectly to the number of cells producing it.

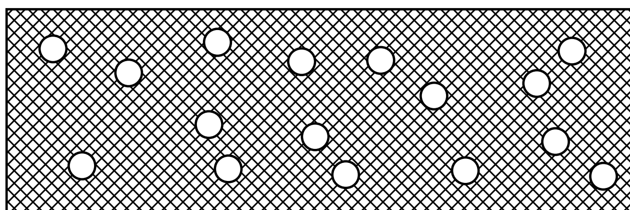
This procedure relies on the use of red blood cells (usually SRBC) as antigen, and on their ability to undergo *lysis* in the presence of antibody and complement. Cells from an immune spleen are mixed with a suspension of SRBC in a warm agarose solution, which is spread in a thin layer on a microscope slide and allowed to solidify. The slides are incubated for one hour, and antibody to SRBC produced by any particular cell in this gel will diffuse and bind to nearby SRBCs. At the end of the hour, the slide is incubated in a suitable dilution of fresh guinea pig serum (as a source of complement), and any SRBCs which are coated with antibody will undergo lysis. The result is a small clear circle (a "hemolytic plaque") on a cloudy background, surrounding each AFC. The single Ab-producing cell in the middle of each plaque is not visible except under a microscope. These clear "plaques", schematically illustrated in Figure A12-1 below (middle slide) can be readily counted, and provide an accurate determination of the number of AFCs: *each antibody-secreting cell produces a single clear plaque.*



No C;
continuous lawn of RBC



C only;
direct (IgM) plaques



C + anti-IgG;
indirect (IgG) plaques,
also including direct plaques

Figure A12-1

Under the conditions described above, only those cells which produce IgM will create a visible plaque; IgG will not generally achieve high enough concentrations to yield two IgG molecules binding sufficiently close together on an SRBC to lyse the cell. In order to detect IgG-secreting cells, antibody to mouse IgG (in this example) is added to the complement solution. Two or more molecules of this *secondary* antibody will then bind to each single IgG bound to an RBC, and will result in lysis in the presence of complement.

The plaques produced in the absence of this secondary "developing" antibody are known as *direct* plaques, and represent the number of IgM antibody producers (*middle* slide above). The additional plaques developed in the presence of the anti-IgG antibody are known as *indirect* plaques and represent IgG producing cells (seen in the *bottom* slide in the figure).

In the example shown above, there are 5 AFCs producing IgM anti-SRBC (direct plaques), and an estimated 11 IgG-producing cells (*i.e.* 16 *total* indirect plaques, from which one must subtract an estimated 5 IgM-producing cells determined from the slide above).

As described here, this technique can only be used to measure those antibody-forming cells producing antibody to red blood cells (SRBC or other RBCs). However, it can be made more generally useful by coupling any desired antigen to RBCs, then using these coated cells as indicator cells for that antigen (similar to what is done for *passive hemagglutination)*. AFCs producing antibody specific for DNP or HGG, for instance, can be detected as plaque-forming cells by using RBCs coated with either the hapten or the protein antigen.

13. HYBRIDOMAS: MONOCLONAL ANTIBODIES

THE PROBLEM: LIMITATIONS OF CONVENTIONAL ANTIBODIES, HETEROGENEITY AND BATCH-TO-BATCH VARIATION

Conventional antisera, as we have seen, contain *complex and heterogeneous mixtures of antibodies* with different antigen-binding specificity, as well as bearing different heavy and light chain isotypes. Immunization of a rabbit, for example, with a human kappa IgG3 myeloma protein will potentially result in formation of antibodies directed against kappa and γ3 isotype epitopes, as well as allotypic and idiotypic epitopes on both heavy and light chains. Note that the rabbit antibodies may themselves consist of a mixture of classes (*e.g.* IgG and IgM) and light chain isotypes (κ and λ), but this heterogeneity is not relevant for our discussion here.

In order to prepare a κ-specific reagent which could be used for a clinical laboratory assay for κ chains, an elaborate and expensive procedure of absorption and/or affinity purification must be carried out, and the resulting antibody extensively characterized for its efficacy and specificity. Furthermore, when this one batch of antibody is used up, the entire procedure must be repeated from scratch, with no guarantee that the next batch will be the same as, or as good as the first, since every new rabbit may respond differently to immunization.

The problems of antibody heterogeneity and batch-to-batch variation are even more serious for those antibodies which are the result of fortuitous "natural" immunization rather than controlled laboratory immunizations, for example those antibodies used for human Ig allotype determination (*e.g.* from polytransfused patients) and for HLA-typing (e.g. from polytransfused patients or multiparous women).

THE SOLUTION: MONOCLONAL ANTIBODIES

In order to resolve these problems it clearly would be desirable to create a source of antibody which is monoclonal (and therefore monospecific), and can be produced in unlimited quantities with no batch-to-batch variation. This is precisely what the development of HYBRIDOMA technology in the mid-1970's has allowed us to do. In principle, we could simply choose a particular antibody-forming cell producing the antibody we desire, and "immortalize" it so that we can propagate it in culture and collect its secreted product indefinitely. Unfortunately these cells cannot ordinarily be grown in culture (although some success has been achieved in immortalizing such cells by transformation with Epstein-Barr virus).

AFC's AND FUSION PARTNERS

We begin with spleen cells from a mouse repeatedly immunized (in this example) with a human κIgG3 myeloma protein. This population is enriched for AFC's producing the Ab we want (anti-κ), although many other irrelevant antibodies will also be represented.

We then harvest cells of a plasmacytoma cell line, the "fusion partner", which has been adapted to cell culture. Several such lines are commonly available, and they have several important properties. First, they have the capacity of unlimited growth in culture.
Second, they have the required machinery for high-level expression and secretion of immunoglobulin; the commonly used lines have also been selected for loss of expression of

their own immunoglobulin, to avoid interference with the specific Ab we want. Third, they have been selected for loss of expression of one of the enzymes required for DNA synthesis by the "salvage pathway". One commonly used fusion partner is the mouse cell line SP2/0, which we will use in this example; it produces no Ig of its own, and is HAT-sensitive (see below).

"HAT" SELECTION

Figure A13-1 outlines the metabolic pathways involved in the "HAT" selection scheme. Cells can produce nucleosides required for DNA synthesis (and therefore cell proliferation) by either of two pathways. First, they can build them up from simple carbon sources such as glycine via the "de novo" pathway indicated as "1"; one step in this pathway requires the enzyme Dihydrofolate Reductase (DHFR), as indicated. Alternatively, if preformed purines and pyrimidines are made available to the cell, it can incorporate them via the "salvage pathway" indicated as "2", one step of which requires the enzyme Thymidine Kinase (TK).

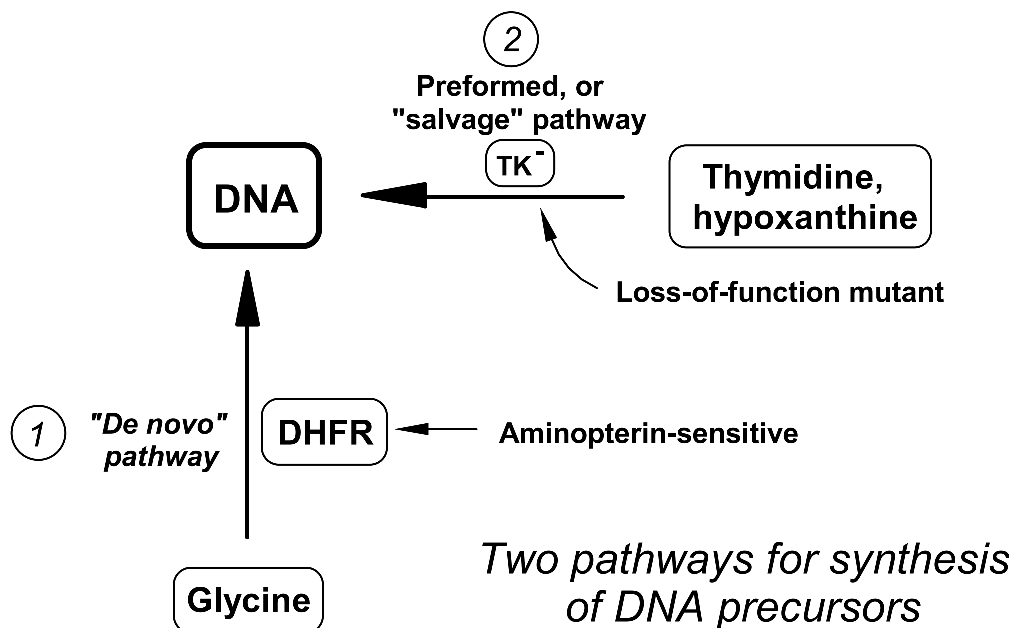


Figure A13-1

However, if a cell has been selected for *loss* of TK expression (indicated in the figure as TK^-), then the cell is completely dependent on the *de novo* pathway in order to synthesize DNA and proliferate. [Selection for loss of other enzymes in this pathway, *e.g.* Hypoxanthine-Guanine Phosphoribosyl Transferase, or HGPRT, can also be used for this purpose]. Such a cell is now exquisitely sensitive to the drug Aminopterin, which is a potent inhibitor of DHFR. This sensitivity is the basis for HAT selection, as discussed below.

MAKING HYBRIDOMAS: FUSION/SELECTION/SCREENING

Three basic steps are involved in the production of the hybridoma we desire.

1) FUSION: Having immunized a mouse with the purified κIgG3 protein, we mix its spleen cells (~10^8 cells) with cells of the fusion partner ("PC" in the figure; ~10^7 cells), in the presence of an agent which facilitates cell fusion (typically either polyethylene glycol [PEG], or Sendai virus). At low frequency (~10^{-5}) individual AFC's (indicated as "B" in the figure) will fuse with individual plasmacytoma cells.

2) HAT SELECTION: The mixture is then distributed into some 1000 separate culture wells (in a number of 96-well plates), and covered with HAT medium. This medium contains Aminopterin ("A"), which blocks the *de novo* synthesis pathway, as well as Hypoxanthine and Thymidine ("H", "T") which allow competent cells to utilize the salvage pathway. As indicated in the Figure A13-2,, *the only cells capable of growing under these conditions are the fusion products*; the unfused plasmacytoma cells are TK^- and are killed by the drug, while the unfused AFCs inherently lack the ability to grow in culture (even though they are TK^+).

3) ANTIBODY SCREENING: After a week or two in culture, a few hundred (say 500) of the wells will contain growing cells, most of them the product of a single fusion event. Supernatants from these 500 wells are then assayed for the presence of Ab of the desired specificity, identifying perhaps ten positive clones. These clones are transferred into larger dishes, subjected to additional rounds of subcloning (to eliminate possible contamination by irrelevant clones or unstable variants), and eventually propagated for collection of the monoclonal antibody they produce, and frozen in liquid nitrogen for long-term storage.

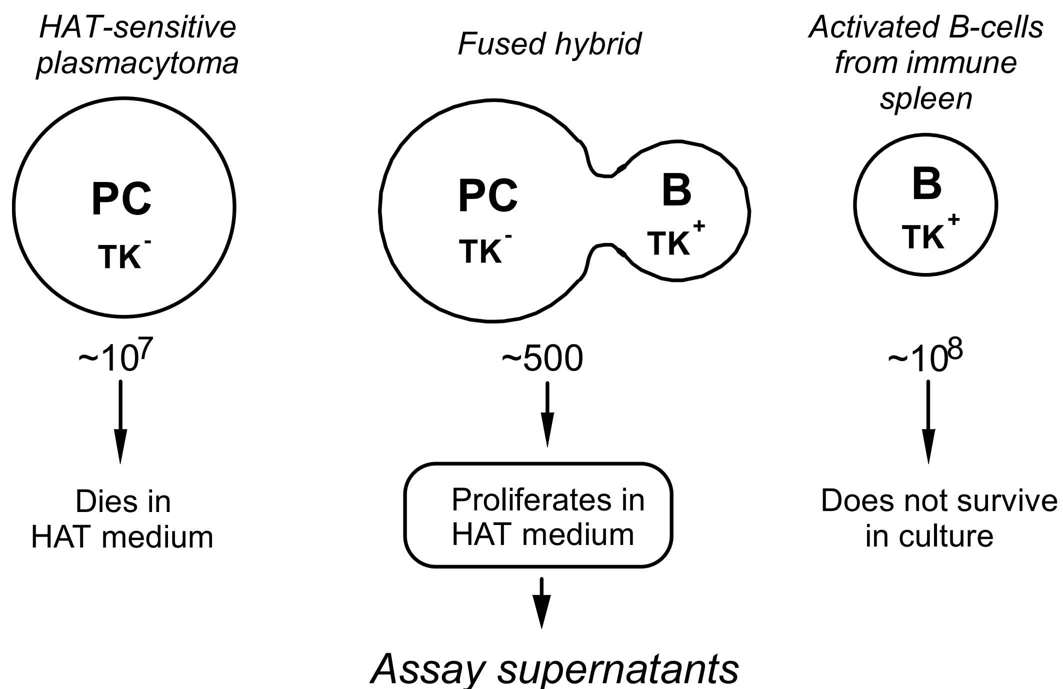


Figure A13-2

In our example, the antibody screening might utilize an ELISA assay with purified κ chain as the antigen; using a variety of different monoclonal κ chains could enable us to identify mAbs with either isotypic or allotypic specificity. [NOTE: In fact, we could have used purified κ chains as the original immunogen, possibly increasing the proportion of useful clones recovered. Two considerations might have led us to immunize instead as described, *i.e.* using the intact κIgG3. First, the time and expense required for κ chain purification are not necessary, since we can readily identify the desired clones even among a large number of irrelevant ones. Second, we might be interested in simultaneously screening the 500 growing clones for anti-γ3(G3)-specific Ab as well, which could be used in other clinically or scientifically relevant assays.]

The final result consists of one or more stable cell lines, each producing a monoclonal antibody specific for human kappa chains, with specificity for either isotype or allotype. These Abs (or the cells producing them) can be shared by laboratories all over the world with the assurance that each lab is using Ab of precisely the same specificity. The Ab produced years later from the same cell line will be identical to the original (so long as rare mutants are avoided), and one batch will have precisely the same specificity as any other batch.

APPLICATIONS

Monoclonal Abs have found widespread use in many areas of clinical and basic relevance, including assays for many drugs and hormones, reagents for blood-typing and (to a more limited extent) HLA-typing, and for defining and diagnosing the presence of different serotypes of pathogenic viruses and bacteria. We've already mentioned (in Chapter 13, "CELL SURFACE MARKERS") the importance of those monoclonal antibodies defining several important markers, *e.g.* CD3, CD4 and CD8, as well as many others. Such antibodies can be used in the laboratory determination of lymphocyte subsets (CD4/CD8), for the depletion of T-cells from potential bone marrow grafts (using anti-CD3, for example), and for immunosuppressive therapy (*e.g.* anti-CD3).

GLOSSARY

ACTIVE IMMUNIZATION: Stimulation of an immune response by exposure to an antigen, *e.g.* injection of a mouse with BSA resulting in anti-BSA antibodies, immunization of people with Measles virus resulting in a protective cell-mediated response. Active immunization results in generation of memory and, therefore, long-lasting immunity, as opposed to PASSIVE IMMUNIZATION.

ADOPTIVE IMMUNITY: Immune responsiveness resulting from ADOPTIVE TRANSER.

ADOPTIVE TRANSFER: The transfer of immune responsiveness from one animal to another by use of immunologically competent cells. This may result in permanent establishment of immune responsiveness, ADOPTIVE IMMUNITY, as opposed to the short-term immunity which follows passive transfer of antibodies. This term commonly refers to experimental systems in inbred animals.

AFFERENT: "Bringing towards"; for example, in the context of an *afferent lymphatic* (a vessel bringing lymph towards a lymph node) or in a more symbolic sense to describe the general processes whereby antigen is brought into contact with the lymphoid system -- thus: "t*he afferent limb of the immune response*." (see EFFERENT.)

AFFINITY: The *association constant* for the equilibrium binding of antibody to an antigenic determinant or hapten; sometimes expressed as the *dissociation* constant. The higher the affinity of a given population of antibodies for a hapten, the lower the concentration of free hapten needed for their binding sites to become occupied. AFFINITY describes the strength of binding of a single antibody combining site with a single hapten; (see AVIDITY)

AGGLUTINATION: The "clumping" of a *particulate* antigen resulting from antibody cross-linking, which may be visible by the naked eye or under a microscope. (see HEMAGGLUTINATION)

ALLELE: Alternative forms of a gene found at the same locus on homologous chromosomes within or between individuals.

ALLELIC EXCLUSION: The process by which only *a single* allele for an immunoglobulin heavy or light chain (of the two which are present for each) is selected for expression in a single B-cell. (Also for some T-CELL RECEPTORS)

ALLERGY: Hypersensitivity state dependent on IgE and mast-cell mediated reactions; IMMEDIATE HYPERSENSITIVITY [In obsolete usage, refers to heightened immune reactivity of any kind to antigen.]

ALLOANTIBODY: Antibody produced in one organism directed against ALLOTYPE determinants of a genetically different individual of the same species. Examples of human alloantibodies include anti-HLA antibodies resulting from transfusions, or anti-Rh antibodies which develop in an Rh-negative mother following her delivery of an Rh-positive baby.

ALLOGENEIC: see ALLOGRAFT

ALLOGRAFT: An ALLOGENEIC tissue graft, *i.e.* one performed between genetically *different* individuals of a single species (*for example,* between two different strains of inbred mouse, or between two unrelated humans). (see also ISOGRAFT and XENOGRAFT).

ALLOTYPE: A genetic POLYMORPHISM or "allelic type" of immunoglobulin. Allotypes can be distinguished by physicochemical and chemical criteria, but are most often defined by *antigenic specificities*. These allotypic specificities vary between different members of the same species, and are inherited in an allelic manner (which distinguishes them from ISOTYPES).

ANAPHYLAXIS: An ALLERGIC reaction characterized by a drastic drop in blood pressure. In clinical medicine this may result from injection of a drug (*e.g.* penicillin) or ingestion of an allergen (*e.g.* peanuts) in sensitive individuals.

ANTIBODY: Immunoglobulins (Ig) produced by lymphoid cells of vertebrates with the ability to specifically bind to antigen. In the absence of known antigen-binding specificity, one should use the more general term *immunoglobulin*.

ANTIGEN: A molecule which is specifically recognized and can be bound by antibody. It is customary to characterize the ability of molecules to react with antibody as their *antigenicity*, while the capacity to actively induce an immune response on injection into the body is referred to as the *immunogenicity* of the molecule. An antigen acting as an inducer of any type of immune response is referred to as an *immunogen*.

ANTIGENIC DETERMINANT: see EPITOPE.

ANTIGEN-PRESENTING CELL (APC): A cell capable of presenting antigenic peptides associated with MHC Class II to a T-cell, and providing the required co-stimulatory signals (*e.g.* by expressing cell surface B7 which can be recognized by the T-cell's CD28). The so-called "professional APC's" constitutively express Class II, and include DENDRITIC CELLS, MACROPHAGES and B-CELLS.

ARTHUS REACTION: An inflammatory (skin) reaction, initiated by *antigen-antibody complexes and complement*, characterized by edema, hemorrhage, PMN infiltration, and necrosis. This term most commonly refers to the experimentally induced skin reaction which follows administration of passive antibody and antigen, but may also be used more generally to refer to *immune complex-mediated tissue damage* (for example, in the kidney glomeruli due to serum sickness or autoantibodies).

ATOPY: ALLERGY.

AUTOGRAFT: A tissue taken from one place in an organism and grafted into another location in the same organism (*e.g.* skin grafts in a burn victim). Note that such grafts are, by definition, ISOGENEIC.

AUTOIMMUNITY: Specific immunity, either humoral or cell-mediated, to constituents of the body's own tissues (autoantigens). If such reactions result in tissue damage sufficient to cause any clinical abnormality, an autoimmune disease is present.

AVIDITY: A measure of *the ability of multivalent antibody to form stable complexes with multivalent antigen*. This depends on AFFINITY, on the valency of the antibody and antigen, and on other features of antigen and antibody structure.

BARE LYMPHOCYTE SYNDROME: Immunodeficiency disorder caused by a genetic defect which prevents the expression of MHC Class I ("Type I"), or Class II ("Type II").

B-CELL: Precursor of antibody-forming cells (AFCP). A lymphocyte derived from the haemopoietic tissues of mammals (bone marrow or fetal liver) or the Bursa of Fabricius in birds, and capable, on antigenic stimulation, of yielding a clone of antibody-forming cells. While all cells actively secreting antibody belong to the B-cell lineage, the unqualified term "B-cell" generally refers to the resting state of the cell *prior to Ag*

stimulation. B-cells may be defined by the property of bearing readily detectable surface immunoglobulin (IgM and IgD) or other B-cell-specific surface antigens.

BCG: *Bacille Calmette-Guerin*, an attenuated strain of the bacterium *Mycobacterium bovis* (the causative agent of bovine tuberculosis). This organism has been used both experimentally and clinically as a non-specific stimulator of the immune system and is used for vaccination against tuberculosis in many parts of the world.

BENCE-JONES PROTEIN: Monoclonal immunoglobulin light chains produced by PLASMACYTOMA tumor cells and excreted into the urine.

BETA-2-MICROGLOBULIN (β2M): A small (12 kD) non-polymorphic protein member of the immunoglobulin superfamily, one of the two polypeptide chains present in *all* MHC Class I molecules.

BGG: Bovine gamma globulin. Often used as a carrier protein for hapten immunization.

BSA: Bovine serum albumin. Often used as a carrier protein for hapten immunization.

CAPPING: The aggregation of particular cell surface molecules into a single mass which may subsequently be shed or internalized. "Capping" may be induced by crosslinking membrane molecules, for example with an antibody.

CARCINOGENIC: Describing a substance or procedure known to be capable of causing an increased incidence of malignancy; thus, a carcinogenic chemical. The word is almost synonymous with oncogenic, though by common usage, chemicals are usually referred to as being carcinogenic, whereas viruses capable of inducing tumors are usually termed oncogenic viruses.

CARRIER: A protein involved in the induction of immune responses when coupled to a hapten. The investigator measures antibody production to the hapten, but may be interested in the role that the carrier protein plays in the cellular phenomena of immunity. Carriers are usually themselves immunogenic, and in most situations the injection of a hapten-carrier complex leads to antibody formation also against the carrier.

CELL MEDIATED IMMUNITY (CMI): Specific immunity which is dependent on T-cells but *not* on antibody; it is expressed in one form as the cutaneous delayed type hypersensitivity (DTH) reaction and is important in defense against infection by viruses, fungi, intracellular bacteria and some protozoa, and in rejection of tissue and organ grafts.

CENTRAL LIMB: Central limb of the immune system, the part involving the triggering and differentiation of *antigen-specific lymphocytes*.

CHEMOTAXIS: The directed movement of cells in a particular direction or to a particular site, as for example the migration of lymphoid cells into a site of inflammation.

CHIMERA: An individual organism composed of two or more populations of cells with different genotypes; usually experimentally created, as in *adoptive transfers*.

CLASS (of Ig): The class of an immunoglobulin molecule is defined by the ISOTYPE of its *heavy chain*; IgG represents the class which bear *gamma* H- chains, IgA bear *alpha* H-chains, etc. Ig molecules of any given class (*i.e. heavy chain class*) may bear *any* of the existing light chain isotypes (*e.g*. kappa or lambda).

CLASS I and II (MHC molecules): see MHC

CLONE: All the cells derived from a single progenitor cell by repeated mitosis, and all having the same genetic constitution (apart from mutation or gene rearrangement).

CLONAL DELETION (=CLONAL ABORTION): The process by which auto-reactive B- or T-cells are destroyed early during their maturation.

CLONAL SELECTION: A hypothesis developed by Burnet in the 1950's by which antigen-precommitted precursors are *selected* by the presence of specific antigen to proliferate and differentiate into immunocompetent cells. Originally proposed for an understanding of B-cell function, but it holds as well for T-cells.

CMI: see CELL MEDIATED IMMUNITY

CODOMINANCE: The phenotypic expression of *both* alleles for a particular locus in a heterozygote. For example, all Ig genes are codominantly expressed in an individual (despite the phenomenon of allelic exclusion in individual B-cells).

COMBINATORIAL ASSOCIATION: In immunoglobulin biosynthesis, the *random* association of light and heavy chain variable regions to form the antigen-binding site of antibodies; a major contributor to diversity (also for T-CELL RECEPTORS).

COMBINATORIAL JOINING: In immunoglobulin gene rearrangement, the random joining of V and J gene segments of light chains (V, D and J segments for heavy chains), a major contributor to diversity (also for T-CELL RECEPTORS).

COMPLEMENT: An enzymatic complex of serum proteins that is activated by many antigen-antibody reactions, and which is essential for antibody-mediated immune hemolysis and bacteriolysis; it also plays a major part in clearance of immune complexes and other biological reactions.

COMPLEMENT FIXATION: Initiation of the COMPLEMENT cascade, resulting in the consumption of active complement. The Complement Fixation Assay is a tool which may be used to detect and quantitate the presence of either antigen or antibody, relying on the ability of (some) antigen/antibody complexes to consume complement.

CR: Complement receptor; a variety of receptors specific for different complement components and their proteolytic products are present on a variety of cells of the immune and hematopoietic system.

CYTOKINE: Any of a large number of secreted proteins mediating signals between populations of leukocytes during immune responses. Includes *lymphokines* produced by lymphocytes (*e.g.* IL-2 and IL-4), and *monokines* produced by monocytes (*e.g.* IL-1 and IL-6)

CYTOPHILIC ANTIBODY: Antibody passively adsorbed on to the surface of a cell which did not produce it (e.g. macrophages, B-cells), bound by cell receptors specific for the Fc fragment of the antibody.

DELAYED-TYPE HYPERSENSITIVITY (DTH): Any manifestation of CELL MEDIATED IMMUNITY (*e.g.* TB skin test), alluding to the fact these reactions usually take a day or more to develop, as opposed to antibody-mediated reactions or HUMORAL IMMUNITY which may be manifested in minutes.

DENDRITIC CELL: Bone-marrow-derived cells with extensive finger-like processes. Immature dendritic cells reside in tissues, take up pathogenic or other antigenic material by phagocytosis, and migrate via afferent lymphatics to a lymph node where they mature and present antigen to T-cells.

DETERMINANT: see EPITOPE.

DNP: The dinitrophenyl HAPTEN, or dinitrophenol.

DOMINANT: Describing an allelic trait which is phenotypically expressed in individuals who are *heterozygous* for the particular allele (as well as in homozygotes); contrasted with the RECESSIVE allelic form, expressed only in homozygotes.

DTH: (see DELAYED-TYPE HYPERSENSITIVITY)

EFFECTOR: Usually used as an adjective, *e.g., effector cell*, which carries out the final stages of an immune reaction. Activated T-cells (for cellular immunity) and plasma cells (for humoral immunity) can both be considered effector cells. (Other cell types can also have effector roles in immune reactions.)

EFFERENT: "Carrying away"; efferent lymphatics carry lymph (and cells) away from lymph nodes. The *efferent limb of the immune response* includes all the effector functions of antibodies and T-cells. (see AFFERENT.)

ELISA: **E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay; any assay for antigen or antibody which relies on detection of enzymed-linked molecules.

EPITOPE: The minimal portion of an antigen molecule which is recongized and bound by an antibody; also referred to as an ANTIGENIC DETERMINANT. Epitopes on the surface of proteins or carbohydrates are small, commonly being in the size range equivalent to 3-6 amino acids.

Fab: "Antigen-binding" fragment of immunoglobulin molecules produced by proteolysis, containing the entire light chain and the N-terminal portion of the heavy chain; includes the variable regions of both chains.

Fc: "Crystallizable" fragment of immunoglobulin molecules produced by proteolysis, contains only the C-terminal portion of the heavy chain constant region.

FcR: "Fc-receptor"; diverse family of receptors specific for the Fc region of different Ig isotypes, present primarily on phagocytic cells (macrophages, PMN's) and B-cells.

FITC: *Fluorescein isothiocyanate*, a chemically reactive derivative of flourescein, readily coupled to free amino groups of proteins to render them fluorescent.

FOLLICLE: Ovoid or spherical collection of densely packed lymphocytes identified in histological sections of lymphoid tissues. A *primary lymphoid follicle* is a rounded aggregation of small lymphocytes (mainly B-cells), and dendritic follicular cells; a *secondary lymphoid follicle* is a more complex structure containing the above two elements together with a nest of rapidly proliferating lymphocytes (mixed B and T cells) known as a GERMINAL CENTER.

GENOTYPE: The *genetic* constitution of an individual which results in the expression of a particular PHENOTYPE.

GERMINAL CENTER: (see FOLLICLE.) Region of cell proliferation within a *secondary follicle*; germinal centers are antigen-dependent and T-dependent in their origin, containing *both* T and B cells. Generation of B-memory cells and Ig somatic mutation occur within germinal centers.

GRANULOCYTE: One of three varieties of blood leukocyte with a segmented nucleus and cytoplasmic granules. Includes polymorphonuclear neutrophils (PMN), eosinophils and basophils.

H-2: The major histocompatibility complex (MHC) of the mouse.

HAPLOTYPE: A combination of allelic forms of several loci inherited as a closely linked unit on a chromosome. A haplotype of HLA, for instance, represents a particular combination of allelic forms of HLA-A, B, C, D, etc.

HAPTEN: A small molecule which by itself cannot stimulate antibody production but which can be recognized and bound by antibody once the antibody has been formed (*i.e.* a hapten is *antigenic* but not immunogenic). A hapten can be rendered immunogenic by being coupled to a CARRIER. Landsteiner, in the early 1900's, introduced the use of small chemically defined structures to add greater precision to the study of immunochemistry.

HEMAGGLUTINATION: AGGLUTINATION involving red blood cells (RBC). ACTIVE hemagglutination results from the binding of antibodies to antigens which are a normal part of the RBC membrane; PASSIVE hemagglutination results from antigens which have been artificially attached to RBC membranes.

HETEROGRAFT: A tissue or organ graft between two individuals of *different species*. This older term has been replaced by *xenograft*.

HETEROTOPIC: see ORTHOTOPIC

HETEROTOPIC: see ORTHOTOPIC.

HETEROZYGOTE: An individual possessing two different alleles at a particular locus on a pair of homologous chromosomes.

HEV: HIGH ENDOTHELIAL VENULE. A specialized small vein, originally known as a POST CAPILLARY VENULE (PCV), which is found in the deep cortex of lymph nodes. This vessel is lined by a high, cuboidal endothelium and is prominently involved in the mechanism of recirculation of lymphocytes. The lining cells have special biochemical features which allow the attachment and migration of B- and T-cells from the circulation into the node.

HINGE REGION: That portion of the immunoglobulin heavy chain which lies between CHI and CHII, and around which the Fab regions can typically flex.

HGG: Human gamma globulin.

HISTOCOMPATIBLE: Two individuals identical in those genes with determine the antigenicity of a tissue graft and will therefore accept tissue grafts made between them (see MHC.)

HLA: The major histocompatibility complex (MHC) of humans (the name derives from *Human Leukocyte Antigen*).

HOMOLOGOUS CHROMOSOMES: Chromosomes which pair during mitosis and contain matching loci (although each may bear different alleles at a particular locus).

HOMOZYGOTE: An individual possessing two identical alleles at one particular locus on a pair of homologous chromosomes.

HSA: Human serum albumin.

HUMORAL IMMUNITY: Specific immunity which is dependent upon antibodies present in the blood and tissue fluids.

HYBRIDOMA: An experimentally produced cultured cell line which secretes a monoclonal, homogeneous antibody. This term generally refers to the product of cellular fusion event between a transformed plasmacytoma in culture, and a normal, activated B-

cell. It can more broadly include fusions involving T-cells, macrophages or other lymphoid cells.

HYPERSENSITIVITY: State of the previously immunized individual in which tissue damage results from the immune reaction to a further dose of antigen. May be antibody mediated (immediate) or cell-mediated immunity (delayed).

IATROGENIC: *"Caused by the healer"*; a condition resulting from an undesirable side-effect of medical treatment.

IDIOTYPE: The antigenic determinant(s) of an antibody molecule unique to its combining site, the particular combination of V_H and V_L.

IMMEDIATE HYPERSENSITIVITY: see ALLERGY

IMMUNE COMPLEX: Antigen-antibody complex. May be present in soluble form, especially in antigen excess, or as a precipitate, especially at equivalence; can be deposited in tissues and cause considerable damage.

IMMUNOASSAY: Any method utilizing specific antigen-antibody reaction of biological material (e.g., complement fixation test, hormone radioimmunoassay, radial immunodiffusion test, passive agglutination test).

IMMUNODEFICIENCY: Deficiency in an organism's ability to mount an immune response, may be either congenital or acquired.

IMMUNOGEN: (see ANTIGEN)

IMMUNOGLOBULIN: Member of a family of proteins each made up of light chains and heavy chains linked together by disulfide bonds. All antibodies are immunoglobulins. Present in serum and body fluids; on electrophoresis mostly migrate as gamma-globulins.

IMMUNOLOGICALLY COMPETENT CELL: Synonymous with *Antigen Reactive Cell*. Clonally pre-committed lymphoid cell capable of generating an immune response after activation by antigen, and includes both T- and B-cells.

INBREEDING: Breeding program which generally involves brother-sister matings for many generations which tends to progressively decrease genetic variability. Fully inbred strains (e.g., of mice or rats) are *homozygous at every locus*, and, in addition, individuals within an inbred strain are *as identical to each other as identical twins*.

INNATE IMMUNITY: The ability of an organism to resist microbial infection by mechanisms which do not exhibit memory or clonal specificity. Skin and mucus barriers, phagocytic cells and COMPLEMENT are all part of the innate immune system.

INTERLEUKIN: (see CYTOKINE)

ISOGENEIC: see ISOGRAFT

ISOGRAFT: An ISOGENEIC or SYNGENIC tissue graft, *i.e.* one performed between genetically identical individuals (for example, between two mice of the same inbred strain). (see also ALLOGRAFT and XENOGRAFT).

ISOTYPE: In relation to immunoglobulins, refers to variants of particular heavy or light chains which are present in *all* individuals of a species as opposed to being inherited in allelic fashion (which distinguishes them from *allotypes). Mu*, *gamma*, and *alpha* (among others) are isotypes of heavy chains, *etc.*, and define the CLASS of Ig. *Kappa* and *lambda* are isotypes of light chains.

J-CHAIN: "Joining chain"; the polypeptide chain covalently attached to the heavy chain of secreted IgM and polymeric IgA during the process of their polymerization. (Not to be confused with the J-SEGMENT).

J-SEGMENT: One of two (or three) genetic elements required to form a complete variable region for antibody light (or heavy) chains during the process of gene rearrangement, together with the V-region (and D-region) gene segment(s); not to be confused with the J-CHAIN. (Also present in T-CELL RECEPTOR)

KLH: Keyhole limpet hemocyanin. Very large and highly immunogenic protein, may be used as a carrier protein for hapten immunization, or alone to generate an antibody response or a DTH skin reaction.

LAD (LEUKOCYTE ADHESION DEFICIENCY): A group of rare immunodeficiency diseases caused by a genetic defect in any one of several adhesion molecules expressed on lymphocytes or PMNs.

LEUKOCYTE: White blood cell, including LYMPHOCYTE, MONOCYTE and GRANULOCYTE.

LOCUS: The position in a chromosome at which a particular gene is located.

LYMPHOCYTE: All cells of lymphoid tissue, blood, or bone marrow which are characterized by the possession of a round nucleus, relatively little cytoplasm, and lack of prominent vacuoles or granules. This was originally a morphological term, which includes cells with many different functions, but it is often used in a more restricted sense to refer specifically to T-cells and B-cells.

LYMPHOKINE: General name for soluble factors secreted by lymphocytes which have biological effects on other cells (see CYTOKINE).

MACROPHAGE: Large *phagocytic* cell, responsible for antigen *processing* and *presentation* to T-cells; key component of afferent limb of the immune response. Radioresistant and adherent cell, similar to blood *monocyte* and skin *histiocyte*.

MEDULLA: This word is used as an anatomical term to describe the inner portion of many organs; in immunology, it usually refers to that deep portion of lymph nodes in which extensive phagocytosis takes place, or the deep portion of the thymus.

MHC: *Major Histocompatibility Complex*, a series of closely linked genes encoding CLASS I and CLASS II MHC molecules. Class I MHC molecules, in combination with bound antigenic peptides, are targets for recognition and killing by cytotoxic T-cells; Class II MHC molecules, in combination with bound antigen, are required for antigen presentation to helper T-cells.

MONOCLONAL: Describing an antibody population derived from the progeny of a single antibody-forming cell precursor. HYBRIDOMAS and myeloma proteins are both examples of monoclonal immunoglobulins.

MONOCYTE: see MACROPHAGE

MONONUCLEAR CELLS: Collectively referring to lymphocytes and monocytes/macrophages, to distinguish them from leukocytes with segmented nuclei (*i.e.* PMN's and other granulocytes).

MONOSPECIFIC: Describing an antibody population specific for a particular antigenic determinant.

MULTIPLE MYELOMA: Plasma cell tumor typically characterized by production of MYELOMA PROTEIN and local destruction of bone tissue.

MYELOMA PROTEIN: A monoclonal immunoglobulin molecule, which may be normal or abnormal in its structure, secreted by a myeloma or other tumor of antibody-forming cells (see PLASMACYTOMA). Myeloma protein heavy chains are found in serum, and free light chains in both serum and urine (BENCE-JONES PROTEIN).

NATURAL KILLER CELLS: Lymphocytes which lack T-cell and B-cell markers, and which are capable of killing target cells based on abnormal expression of MHC molecules. Also referred to morphologically as "large granular lymphocytes".

NK CELLS: see NATURAL KILLER CELLS

ONCOGENIC: Describing a substance or procedure known to be capable of causing an increased incidence of malignancy; thus, a carcinogenic chemical. The word is almost synonymous with *carcinogenic*, although by common usage, chemicals are usually referred to as being carcinogenic, whereas tumor-causing viruses are usually termed *oncogenic*.

OPSONIZATION: The facilitation of phagocytosis by macrophages or granulocytes resulting from antibody and/or complement bound to a target (*e.g*. a bacterium).

ORTHOTOPIC: A term describing some tissue transplants. A graft is *orthotopic* if it is placed into the same anatomical region as the organ which it is designed to replace, otherwise it is *heterotopic*. Thus, human heart transplants are orthotopic, whereas human thymus transplants into the abdominal muscle wall are heterotopic.

OVA: Ovalbumin, albumin of chicken (or other birds') eggs. Often used a carrier protein for hapten immunization

PASSIVE IMMUNIZATION: Transfer of immunity by transfer of specific antibody. Injection of antibody to tetanus toxoid, for example, results in short-term resistance to tetanus infection, which decays with the half-life of serum immunoglobulin (~2-3 weeks).

PFC: see PLAQUE-FORMING CELL

PHAGOCYTOSIS: Internalization of extracellular material by the formation of pseudopods and subsequent engulfment; macrophages and PMN's are phagocytic. (see also PINOCYTOSIS and RECEPTOR-MEDIATED ENDOCYTOSIS).

PHENOTYPE: The physical or chemical manifestations of an organism's GENOTYPE. A person whose *phenotype* is blood group A, for example, could be of *genotype* A/A or A/O.

PINOCYTOSIS: Internalization of soluble extracellular material by formation of small membrane vesicles. (see also PHAGOCYTOSIS and RECEPTOR-MEDIATED ENDOCYTOSIS.

PLAQUE-FORMING CELL: Antibody-secreting cell visualized by its ability to cause localized lysis of red blood cells (thus forming a "plaque") in an experimental assay system.

PLASMA CELL: The major immunoglobulin-secreting cell type and the terminally differentiated cell of the B-cell series. It is present within lymphoid organs but rarely in the blood (despite its name), and is characterized by plentiful basophilic cytoplasm with much endoplasmic reticulum and a high content of RNA.

PLASMA: The fluid portion of anti-coagulated blood remaining after the "formed elements" (cells and platelets) are removed. Commonly used anti-coagulants include heparin, citrate, and EDTA (see also SERUM).

PLASMACYTOMA: A tumor derived from a plasma cell, with which it shares morphological characteristics, often secreting either an intact or aberrant MONOCLONAL IMMUNOGLOBULIN.

PMN: Polymorphonuclear neutrophil, the most abundant of the granulocytes in blood, whose cytoplasmic granules stain with neutral dyes.

POLYMORPHIC: Describes a gene or its protein product which exists in distinct allelic forms. The alpha chains of MHC Class I molecules (HLA-A, B and C), for example, are highly polymorphic, although their β2-microglobulin component is not. Other examples of polymorphism are the ABO and Rh blood groups, and ALLOTYPES of immunoglobulin light and heavy chains.

POST CAPILLARY VENULE: see HEV.

PRECIPITATION: The formation of an insoluble complex from a *soluble* antigen by antibody-binding.

RBC: *Red blood cells*. This abbreviation is often preceded by a letter indicated the species from which they are derived; thus, SRBC are sheep erythrocytes, HRBC are from human, BRBC from burros, *etc.*

RECEPTOR: Cell membrane constituent which binds a specific ligand (*e.g.* complement receptors, Fc receptors, *etc*.)

RECEPTOR-MEDIATED ENDOCYTOSIS: Internalization of extracellar macromolecules induced by binding to specific cell-surface receptors. (see also PHAGOCYTOSIS and PINOCYTOSIS).

RECESSIVE: Referring to an allele which is phenotypically expressed only in individuals who are homozygous for the particular allele, and not in those who are heterozygous at the locus. (see DOMINANT).

RED PULP: That portion of the spleen in which erythrocytes are stored, phagocytosed and degraded, in which phagocytosis of foreign material occurs, and where extensive erythro-myelopoiesis occurs in some species (rodents, but not humans). Distinguished from WHITE PULP.

RES: *Reticuloendothelial system*, including the lymphoid tissues and the phagocytic compartments of the vasculature, especially the liver and lungs.

RIA: *Radioimmunoassay*; any antibody-based assay (for detecting and quantitating antigen or antibody) which relies on the use of radioactively labeled antibody or antigen.

ROSETTES: A lymphoid cell which binds a ring of erythrocytes around itself, forms a structure identified microscopically as a *rosette*. Human T-cells bind sheep red blood cells via their CD2 molecules, forming *E-rosettes* (for "erythrocyte" rosette). Erythrocytes coated with IgG antibody can similarly form rosettes with Fc-receptor-bearing cells such as macrophages, which are known as "EA" rosettes.

SEROLOGICAL: Having to do with serum or its components; describing assays or procedures which rely on the use of antibodies (ultimately derived from serum).

SERUM: The fluid portion of blood which remains after a blood clot is formed. Serum is similar in its composition to PLASMA, except for the lack in plasma of those proteins consumed or altered during the process of coagulation.

SRBC: Sheep red blood cells, a commonly used antigen in experimental systems because of the variety of assays in which it can function (agglutination, lysis, PFC assays).

SUPERANTIGEN: A molecule capable of cross-linking an MHC Class II molecule with the variable regions of a large proportion of TCRs (typically activating 2-20% of all T cells). Such molecules, produced by various microorganisms, can result in conditions such as Toxic Shock Syndrome.

SYNGENEIC: see ISOGRAFT.

T-CELL: Thymus derived lymphocyte, responsible for Cell-Mediated Immunity (CMI).

T-CELL RECEPTOR (TCR): The antigen-binding receptor of T-cells; encoded by a family of genes distinct from (but homologous to) immunoglobulins, and produced by the same processes of gene rearrangement. The TCR can recognize antigenic peptides only in the context of MHC Class I or Class II molecules.

TAP *("Transporters associated with Antigen Processing")***:** Part of the machinery by which peptides from endogenously produced proteins are translocated from the cytosol into the ER. Within the ER they become associated with newly synthesized MHC Class I molecules which are in turn transported to the plasma membrane.

TCR: see T-CELL RECEPTOR.

THY-1: A cell surface marker for mouse T-cells, originally known as "theta" antigen. Different strains of mice express different allelic forms of this molecule, and anti-Thy-1 antibodies have been used to distinguish T-cells in adoptive transfer experiments.

TISSUE TYPING: The identification of histocompatibility antigens most commonly MHC. HLA (human MHC) typing is carried out by observing the cytotoxic effect of specific antisera on blood leukocytes collected from the prospective donor and recipient of organs or tissues that are to be transplanted.

TOLERANCE: (Immunological) Inability to mount an immune response *against a specific antigen* which is normally immunogenic, as in tolerance to *self antigens*. Development of tolerance may follow contact with antigen in fetal or early postnatal life or, in adults, administration of very high or very low doses of certain antigens, and can be expressed at the level of T-cells or B-cells, or both.

VASOACTIVE: Having the ability to cause dilation of blood vessels, as for example COMPLEMENT components C3a and C5a.

WHITE PULP: The portion of the spleen containing dense aggregation of lymphocytes, namely lymphoid follicles, and periarteriolar white sheath; distinguished from RED PULP.

XENOGENEIC: see XENOGRAFT

XENOGRAFT: A XENOGENEIC tissue graft, *i.e.* one performed between individuals of different species. (see also ALLOGRAFT and ISOGRAFT).

INDEX

A2m ... 49
ABO blood groups ... 77, 82
absorbed antiserum ... 48
absorption ... 181
acetylcholine receptor ... 145
acquired immunodeficiencies ... 147
Acquired Immunodeficiency Disease (AIDS) ... 151
activated macrophages ... 103
active immunity ... 10
active immunization ... 163
acute disseminated encephalomyelitis . 144
adaptive immunity ... 3
ADCC ... 93
ADEM ... 144
adjuvant ... 9, 14, 165
adoptive transfer ... 55, 84, 106, 108
AFCP ... 54
affinity ... 19, 20, 189, 192
affinity chromotography ... 48
affinity maturation ... 32, 53, 58
affinity purification ... 181
AFP ... 170
agglutination ... 11
AIDS ... 151
alkaline phosphatase ... 177
allelic exclusion ... 53, 65, 69
allergen ... 153
allergic reaction ... 165
allergy skin test ... 154
allograft rejection ... 83, 101
allotype ... 46, 49
alpha-fetoprotein ... 170
ALS ... 151
alternate splicing ... 64
alum precipitation ... 15, 165
anaphylotoxin ... 34
anergy ... 140
antibody ... 6, 9, 11, 19
antibody combining site ... 19
antibody dependent cell-mediated cytoxicity ... 93
antibody heterogeneity ... 20, 191
antigen ... 9, 13
antigen sequestration ... 135
antigen-binding sites ... 23
antigenic competition ... 166
antigenic determinant ... 9
antigenic variation ... 166
antigenicity ... 9
antigen-presenting cell ... 117
anti-HLA antibodies ... 133
antisera ... 47
APC ... 117
appendix ... 123
Arthus reaction ... 34, 154
asthma ... 156
attenuated organisms ... 163, 164
ATxBM ... 107
autoimmune thyroiditis ... 144
autoimmunity ... 5, 143
automimmune diseases ... 41
avidity ... 19, 20, 192
B cell ... 106, 125
bacteriolysis ... 34
Bence-Jones proteins ... 27, 46, 47
binding sites ... 191
biological clearance ... 42
blocking factors ... 172
blood transfusions ... 89
bone marrow ... 123, 129, 130
bone marrow transplants ... 89
BRBC ... 57
bronchoconstriction ... 156
brother sister inbreeding ... 201
Bruton's agammaglobulinemia ... 132, 148
BSA ... 13
Bursa of Fabricius ... 105, 109
capillary dilation ... 156
carcinoembryonic antigen ... 170
carcinogens ... 170
cardiovelofacial syndrome ... 149
carrier effect ... 119
CD antigens ... 115
CD2 ... 111
CD3 ... 111
CD4 ... 111, 114
CD5 ... 111
CD8 ... 111, 114
CDR ... 28

CEA.. 170
cell surface markers 111
cell-mediated
immunity (CMI).... 83, 85, 91, 105, 154
cellular immune responses....................... 6
central limb 147
central lymphoid organs....................... 109
chemotaxis 34
chronic granulomatous disease 147
circulating immune complexes 146
class I MHC 83, 87, 96
class II MHC 83, 87, 96, 117
class switching 65, 69
classes of Ig..................................... 23, 25
classical complement pathway.............. 35
class-switching...................................... 61
clonal abortion 58, 135, 139
clonal expansion.............................. 55, 58
clonal selection..................... 32, 53, 54, 57
CMI 83, 91
Cohn fractionation 17
combinatorial association................ 61, 67
combinatorial joining....................... 61, 66
complement... 33
complement deficiencies...................... 148
complement fixation assay................... 197
complement receptor (CR)............. 12, 112
complementarity determining
regions (CDR)......... 28
concanavalin A (ConA) 115
concomitant immunity 172
congenital immunodeficiencies............ 147
congenital thymic aplasia..................... 149
constant region 23, 27
constant region 45
contact dermatitis 92
CR 12, 34, 111, 112
CR-bearing pre-B-cells 149
cross-matching 78
cross-reactivity 195
cyclosporin 152
cytolysis.. 12
cytotoxic drugs 151
cytotoxic T cells 91
cytotoxicity.. 12
degranulation....................................... 156
delayed type
hypersensitivity (DTH) 153, 154
diffuse cortex 123
DiGeorge syndrome 149
diphtheria 164, 167
direct plaques 208
diversity.. 53
dizygotic cattle twins 135
DNA rearrangement.............................. 62
DNP... 13, 120
domains 28
DTH response 91, 115
EAE.. 145
ECF-A 156
electron microscope 177
electrophoresis 17
ELISA 177, 184, 187
encephalomyelitis................................ 143
endogenous peptides 98
enhancing antibodies............. 85, 132, 171
enteropathies 151
enzyme labeling 177
enzyme-labeled antibodies.................. 178
eosinophil 156
eosinophilia 156
epitope... 9
equilibrium constant.................... 189, 190
equilibrium dialysis....................... 19, 189
erythroblastosis fetalis..................... 77, 81
ethanol precipitation.............................. 17
exchange transfusion............................. 81
exogenous peptides 98
experimental allergic
encephalomyelitis.......... 145
eye lens protein 143
F(ab) 24
$F(ab')_2$ 24
F_1 inbred crosses................................ 203
Fc.. 24
FcR....................................... 12, 111, 112
Fc-receptors.................................. 12, 112
fetal development................................ 131
fetal liver 129
fetal thymus transplant 149
first set rejection.................................... 83
fluorescence-activated cell
sorter (FACS)............... 177
fluorescent labeling 177
Freund's adjuvant.................................. 14
GALT 123

gamma-globulin 19
gene rearrangement 61
generation of diversity...................... 61, 66
germinal centers 123, 125, 127
germ-line theory 61
Goodpasture's syndrome 146
graft rejection 5, 84, 91
graft versus host (GvH)...... 83, 88, 92, 149
H-2 86
haplotype exclusion............................... 65
hapten 13, 119
Hashimoto’s thyroiditis........................ 144
heavy chain class switching 65
heavy chains 23, 24, 61
hemagglutination................................... 11
hematopoietic stem cells 129
hemolysis 12, 154
heterologous antibodies 165
high endothelial venules (HEV)........... 125
hinge region... 29
histamine 156
histocompatibility antigens 86
HIV.. 151
HLA 86
homocytotropic 155
horseradish peroxidase......................... 177
hot antigen suicide 55
^{3}H-TdR suicide...................................... 56
Human Immunodeficiency Virus (HIV) 151
humoral immunity............................. 6, 85
H-Y antigen... 137
hybridoma 114
hyperimmune rejection 85
hypervariable regions 27
hypocalcemic tetany............................. 149
iatrogenic... 147
identity – non, partial, complete........... 179
idiotype.. 46
IF, immunofluorescence....................... 177
IFN-γ 96
IgA, IgD, IgE, IgG,......................... 25, 26
IgM 25, 26, 193
IL-17.. 114
IL-2.. 96
IL-4.. 96
immobilization 12
immune adherence 12, 34
immune clearance 42
immune complex disease ... 33, 34, 41, 154
immune complexes 41, 143, 154
immune response (Ir) genes 87
immunoadsorption 181
immunodeficiency................................ 135
immunodiagnosis 172
immunofluorescence (IF) 177
immunogen 9, 13
immunoglobulin 19
immunoglobulin genes 61
immunological enhancement 85
immunological memory 23, 30, 84
immunological surveillance 170
immunologically privileged sites ... 84, 141
immunomodulation 169
immunoselection 169
immunosorbent assay (ELISA) 11
immunosuppression 135
immunotherapy 172
in vitro primary antibody response 56
inbreeding 201
inflammation 3
influenza.. 167
innate immunity 3
intracellular parasites 91
intravenous 157
intravenous IgG (IVIG)........................ 149
ionizing radiation 152, 170
Ir genes 87, 101
isoagglutinins 77
isograft 83
isotype 23, 25, 46, 49
J-chain 69
J-segments... 64
kappa chain complex............................. 62
kappa chains 25, 46, 61, 66
Kern... 50
kidney.. 146
killed organisms 164
kinins... 156
Km... 46
LAK 173
lambda chains...................... 25, 46, 50, 61
large granular lymphocytes 93
latent period 158
LCMV 96
leader polypeptide 64

lectins 115
leukotrienes 156
light chains 23, 24
Listeria 102
luciferase 177
luminescence 178
lupus erythematosus 41
lymph node 123
lymphatic 125
lymphocyte-determined (LD) 87
lymphocytes 154
lymphoid system 6
lymphoid tissue 123
lymphokine-activated killer (LAK) 173
macrophage 91, 117, 154
major histocompatibility
complex (MHC) 83, 86
malignancies 151
marginal sinus 125
mast cells 153
maternal IgG 131, 167
maternal immunization 129
Mcg 50
memory 4, 53
memory cells 58
metastasis 169
MHC 83, 86
MHC class I & II 96, 111, 112
MHC restricted recognition 91, 94
mIg 111
migration 126, 130
mitogens 115
mitomycin treatment 205
mixed lymphocyte reaction (MLR) 92
monoclonal gammopathy 20
mononuclear cells 154, 155
mucus secretion 156
multiple sclerosis 145
muramyl dipeptide 15
myasthenia gravis 143, 145
mycobacterium 14, 166
myeloma 20, 26, 192
natural antibodies 53, 59, 79
natural killer (NK) cell 93
neo-antigens 169
neoplasia 169
neutrophils (PMN) 154
nude mouse 105, 109
oncofetal antigens 170
oncogenes 169
one-shot serum sickness 42
one-way MLR 205
opsonization 12, 34, 112
ouchterlony 11
Ouchterlony 46, 179
papain 24
passive agglutination 11
passive cutaneous anaphylaxis (PCA) . 153
passive immunity 10, 163
PCA 153
pepsin 24
pertussis 167
Peyer's patches 123
PFC 106, 207
phagocytosis 147
pharyngeal pouches 149
plaque-forming cell (PFC) 106, 207
PMNs 154
pneumococcus 11
Pneumocystis carinii 149
point of equivalence 175
pokeweed mitogen 115
poliomyelitis 167
polyclonal activation 60
post-translational processing 69
Prausnitz-Küstner 153, 158
precipitin curv*e* 175
primary follicles 123
primary immunodeficiencies 147
proteolytic fragments 23
radioimmunoassay (RIA) 11, 177
radiolabeling 177
rashes 157
reaginic antibody 155
reagins 155
receptor blockade 135, 139
recirculation 123, 125, 126
renal failure 151
RER 64
reticuloendothelial system (RES) 123
Rh 77
Rh incompatibility 129
rheumatoid arthritis 41, 143, 145
rheumatoid factor 145
rhinitis 157
RhoGam therapy 81

RIA 177, 184, 185
RNA splicing 64
rough endoplasmic reticulum................. 64
Sabin polio vaccine 166
salt precipitation.................................... 17
SCID 150
second set rejection 84
secondary follicle 125
secondary immune responses........... 23, 30
secondary immunodeficiencies 147
selective IgA deficiency 150
self-renewal... 129
self-tolerance....................................... 135
sequestered antigens............................ 143
serologically setermined (SD)................ 87
serum sickness 33, 163, 165, 184
severe combined immunodeficiency.... 150
signal 64
skin grafts.. 83
SLE 146
smallpox... 167
somatic mutation............................. 61, 66
somatic theory....................................... 61
Southern blot.. 199
specificity... 4, 84
spermatozoa antigens 143
spleen 123, 125
SRBC 57
SRS-A 156
stable complexes 192
stem cells... 130
steroids 151
subclasses.. 25
superantigen 100
suppression.. 135
suppressor cells 138
syngeneic... 83
systemic lupus erythematosus (SLE) ... 146
T cell 106, 125
T cell receptor (TCR)............................. 67
TATA 170
T_C 113
T_C cell 91, 94, 111
T_C-mediated lysis.................................. 92
TcR... 111, 112
tetanus 164, 167
T_H1 & T_H2 .... 91, 94, 96, 111, 113
T_H17 .... 111, 113
thoracic duct............................... 107, 125
thymectomy... 105
thymic transplant................................. 150
thymus.. 105, 123
thymus-dependent (TD) antigen 105
thymus-dependent area......... 108, 123, 125
thymus-independent (TI) antigens122, 127
thymus-independent area 108, 123
thyroglobulin....................................... 143
TILs 173
tingible body macrophages 125
tissue mast cell 155
tolerance.................... 14, 53, 58, 129, 135
tonsils 123
toxoids... 164
transcription 64
transfusion reaction 78
translation.. 64
transplantation....................................... 85
transplantation antigens 79
T_{reg} 111, 113
TSTA... 170
tuberculin skin test 91, 154
tumor 169
tumor suppressor genes....................... 169
tumor-associated antigens 170
tumor-infiltrating leukocytes............... 173
tumor-specific antigens 170
two-way MLR 205
type I, II, III, IV reactions............ 153, 154
umbilical cord blood 132
universal donor & recipient................... 78
vaccination 163
valency 19
variable region................................ 23, 27
variolation 163
vascular anaphylaxis 157
vasoactive.. 34
vasodilation 156
venule permeability............................. 156
viruses 170
wheal and flare 158
X-linked infantile
hypogammaglobulinemia................. 148
yolk sac 130