Principles, Synthesis and Applications of monoclonal antibodies

CONTENTS

1. INTRODUCTION

IMMUNE SYSTEM:

The immune system is a host defense system comprising many biological structures and processes within an organism that protects against disease.

The immune system protects organisms from infection with layered defenses of increasing specificity .In simple terms, physical barriers prevent pathogens such as bacteria and viruses from entering the organism. If a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response. Innate immune systems are found in all plants and animals. If pathogens successfully evade the innate response, vertebrates possess a second layer of protection, the adaptive immune system, which is activated by the innate response. Here, the immune system adapts its response during an infection to improve its recognition of the pathogen. This improved response is then retained after the pathogen has been eliminated, in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger attacks each time this pathogen is encountered.

TYPES OF IMMUNE SYSTEM

NON-SPECIFIC DEFENCES (INNATE IMMUNITY)		SPECIFIC DEFENCES (ADAPTIVE IMMUNITY)
First line of defense	Second line of defense	Third line of defense
• Skin • Mucous membranes • Secretions of skin and mucous membranes	• Phagocytic leukocytes • Antimicrobial proteins • Inflammatory response • Fever	• Lymphocytes • Antibodies • Memory cells

INNATE IMMUNITY

Innate immunity refers to nonspecific defense mechanisms that come into play immediately or within hours of an antigen's appearance in the body. These mechanisms include physical barriers such as skin, chemicals in the blood, and immune system cells that attack foreign cells in the body.

ADAPTIVE IMMUNITY

The adaptive immune system, also known as the acquired immune system or, more rarely, as the specific immune system, is a subsystem of the overall immune system that is composed of highly specialized, systemic cells and processes that eliminate pathogens or prevent their growth. The acquired immune system is one of the two main immunity strategies found in vertebrates (the other being the innate immune system).

Adaptive immunity is further divided into two types

Humoral immunity

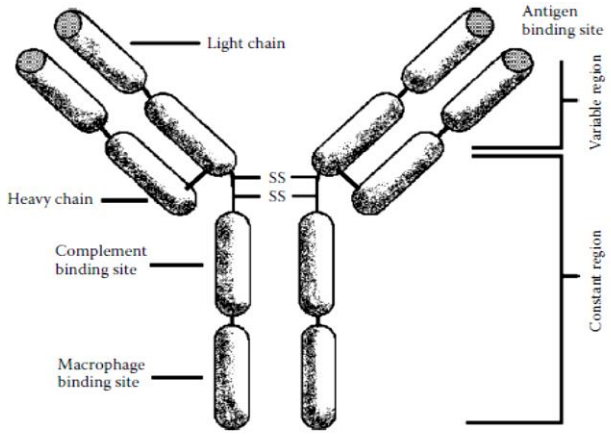
Humoral immunity is the aspect of immunity that is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins, and certain antimicrobial peptides. Humoral immunity is so named because it involves substances found in the humors, or body fluids. It contrasts with cell-mediated immunity. Its aspects involving antibodies are often called antibody-mediated immunity.

Cell mediated immunity

Cell-mediated immunity is an immune response that does not involve antibodies. Rather, cell mediated immunity is the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to antigen.

ANTIBODY

An antibody (Ab), also known as an immunoglobulin (Ig),is a large, Y-shaped protein produced mainly by plasma cells that is used by the immune system to neutralize pathogens such as pathogenic bacteria and viruses. The antibody recognizes a unique molecule of the pathogen, called an antigen, via the fragment antigen-binding (Fab) variable region.



MONOCLONAL ANTIBODY

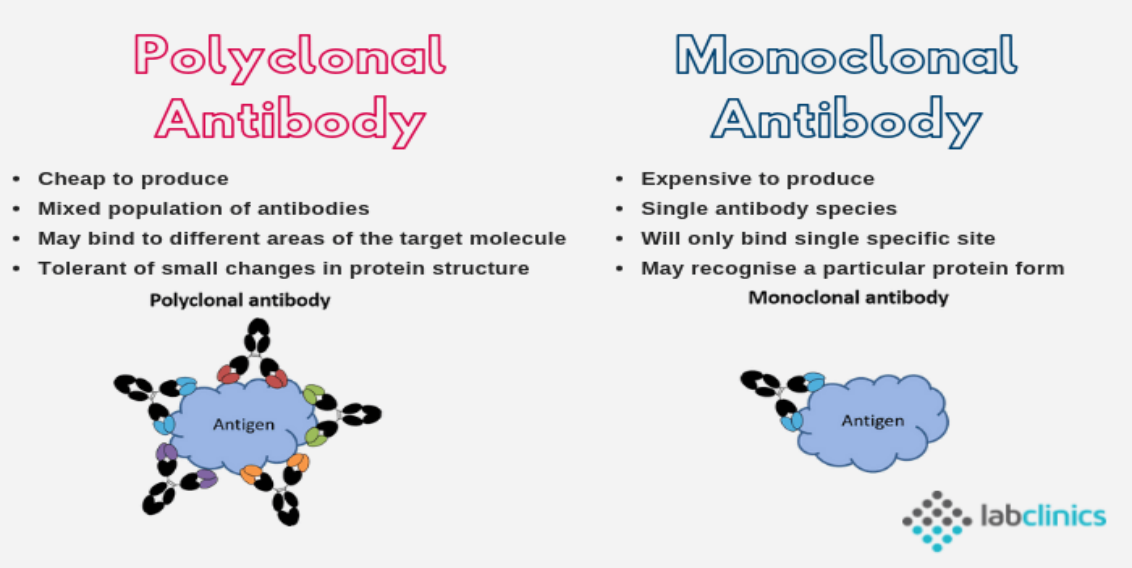
Monoclonal antibodies (mAb or moAb) are antibodies that are made by identical immune cells that are all clones of a unique parent cell. Monoclonal antibodies can have monovalent affinity, in that they bind to the same epitope (the part of an antigen that is recognized by the antibody).

Given almost any substance, it is possible to produce monoclonal antibodies that specifically bind to that substance; they can then serve to detect or purify that substance. This has become an important tool in biochemistry, molecular biology, and medicine.

2. HISTORY

1900. Paul Ehrlich, who is regarded as one of the fathers of modern immunology, proposed the sidechain theory where he hypothesized that side chain receptors on cells bind to a given pathogen. He was the first to propose a model for an antibody molecule in which the antibody was branched and consisted of multiple sites for binding to foreign material, known as antigen, and for the activation of the complement pathway.
1973.
. Over the last three decades, monoclonal antibodies (MAbs) have made a striking transformation from scientific tools to powerful human therapeutics. Muromonab CD3 a murine MAb was the first FDA approved therapeutic MAb for the prevention of kidney transplant rejection. Since its approval in 1986, there has been a decline in further application and approvals until the late 1990s when the first chimeric Mab, Rituximab was approved for the treatment of lowgrade B cell lymphoma in 1997. With the approval by licensing authorities of chimeric, followed by humanized and then fully human monoclonal antibodies, the rate of approval and monoclonal antibodies available in the market for the treatment of various diseases has increased dramatically. As of March 2017, FDA has approved approximately 60 therapeutic MAbs which are currently under evaluation in various phases of clinical trials

3.DIFFERENCE B/W POLYCLONAL AND MONOCLONAL ANTIBODIES

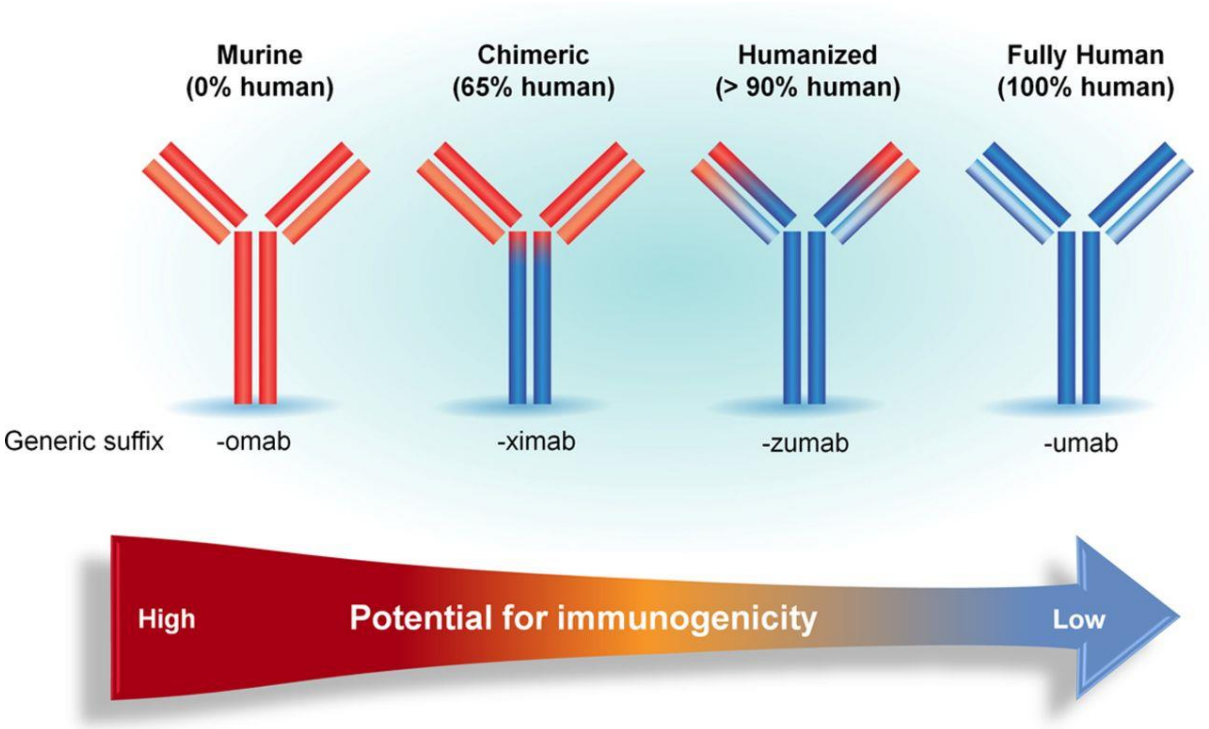


4. CLASSIFICATION OF MONOCLONAL ANTOBODIES

Monoclonal antibodies are classified on basis of:

1.On basis of origin

1. Murine: Both the chains are of mouse origin. E.g. muromonab CD3 and edrecolomab. Total number of murine antibodies marketed are 5.
2. Chimeric: A chimeric antibody is one of which both chain types are chimeric as a result of antibody engineering. A chimeric chain is a chain that contains a foreign variable domain (originating from one species other than human, or synthetic) linked to a constant region of human origin. Usually 65% of total structure is of human origin. E.g. abciximab and rituximab.
3. Humanized: A humanized antibody is one of which both chain types are humanized as a result of antibody engineering. A humanized chain is a chain in which the complementarity determining regions (CDR) of the variable domains are foreign (originating from one species other than human, or synthetic) whereas the remaining chain is of human origin usually 95% portion is of Human origin. e,gTrastuzumab and omalizumab
4. Human : A human antibody is one of which both chain types totally are of human origin. Eg adalimumab and Ofatumumab. 22 human mABs are in market.



B. On basis of Applications

1. Diagnostic antibodies: USE as diagnostic aid for example Humaspect (Votumumab) used for diagnosis of

colorectal carcinoma
2. Therapeutic antibodies: Therapeutic (Mab) eg omalizumab inhibitor of IgE receptors used for severe persistent asthama

5. NOMENCLATURE

Barone's Guide to Monoclonal Antibody Nomenclature

How to name a monoclonal antibody:

MABs are named by combining a **Prefix** + **Target** + **Source** + **Suffix** (usually "mab")

Prefix	+	Antibody Target		+	Antibody Source		+	-mab
		• Bone	-o(s)-		• Human	-u-		
		• Cardiovascular	-c(i)-		• Humanized	-zu-		
		• Immune-modulating	-l(i)-		• Chimeric	-xi-		
		• Interleukin	-k(i)-		• Rat	-a-		
		• Tumor	-t(u)-					
		• Virus	-v(i)-					

Examples

Classic Examples	Target	Source
Den-*o*-*su*-mab	Bone: RankL	• Human
Beva-*ci*-*zu*-mab	Cardiovascular: VEGF	• Humanized
Ab-*ci*-*xi*-mab	Cardiovascular: GP IIb/IIIa	• Chimeric
Inf-*li*-*xi*-mab	Immune-modulating: TNF	• Chimeric
Ri-*tu*-*xi*-mab	Tumor: B-cell CD20	• Chimeric
Tras-*tu*-*zu*-mab	Tumor: HER2-neu receptor	• Humanized
Pali-*vi*-*zu*-mab	Virus: Fusion protein of RSV	• Humanized

6. PRODUCTION OF MONOCLONAL ANTIBODIES

Monoclonal antibodies (mAbs) are the dominant group of recombinant proteins used in human therapy. These proteins were first successfully developed by Köhler and Milstein by using HYBRIDOMA TECHNOLOGY in mid-1970s and published in 1975.

A.HYBRIDOMA TECHNOLOGY

The mAbs have been developed by using mouse hybridoma technology for therapeutic applications. This technique uses innate functionality of B-lymphocytes and myeloma or immortalized cancerous cells for the effective production of immortal hybridoma cells secreting mAbs specific for antigen of interest. The B-cells are fused with myeloma cells in the presence of polyethylene glycol (PEG) as a fusing agent . The antibodies produced by this technique are specific in nature to the target antigen and has various application in therapeutics.

However, immune responses are often weak in mice, resulting in low affinity and/or non-specific mAbs. Animals other than rodents have not been usually used to produce mAb due to the problems associated in the establishment of immortalized antibody-producing cell lines by hybridoma .

Procedure:

1.IMMUNIZATION OF ANIMAL

Laboratory animals like mice are administered with a series of injections of the test antigen for several weeks against which the antibody is to be generated.

2.CELL FUSION

The spleenocytes are isolated from the mouse spleen and the B cells are fused with immortalised myeloma cells by the process of electrofusion. Alternatively, chemical reagent like polyethylene glycol is used for the fusion of B-cells and myelomas. The myeloma cells are selected which lack the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene, making them sensitive to the HAT medium.

3.SELECTION OF HYBRIDOMAS

The fused cells are incubated in HAT medium(hypoxanthine-aminopterin-thymidine medium)for about 10 to 14 days. The unfused myeloma cells are removed as they can outgrow other cells,especially weakly established hybridomas. Unfused B cells die as they have a short life span. By this method the B cell-myeloma hybrids survive.

4.ISOLATION OF MONOCLONAL ANTIBODIES WITH SINGLE SPECIFICITY

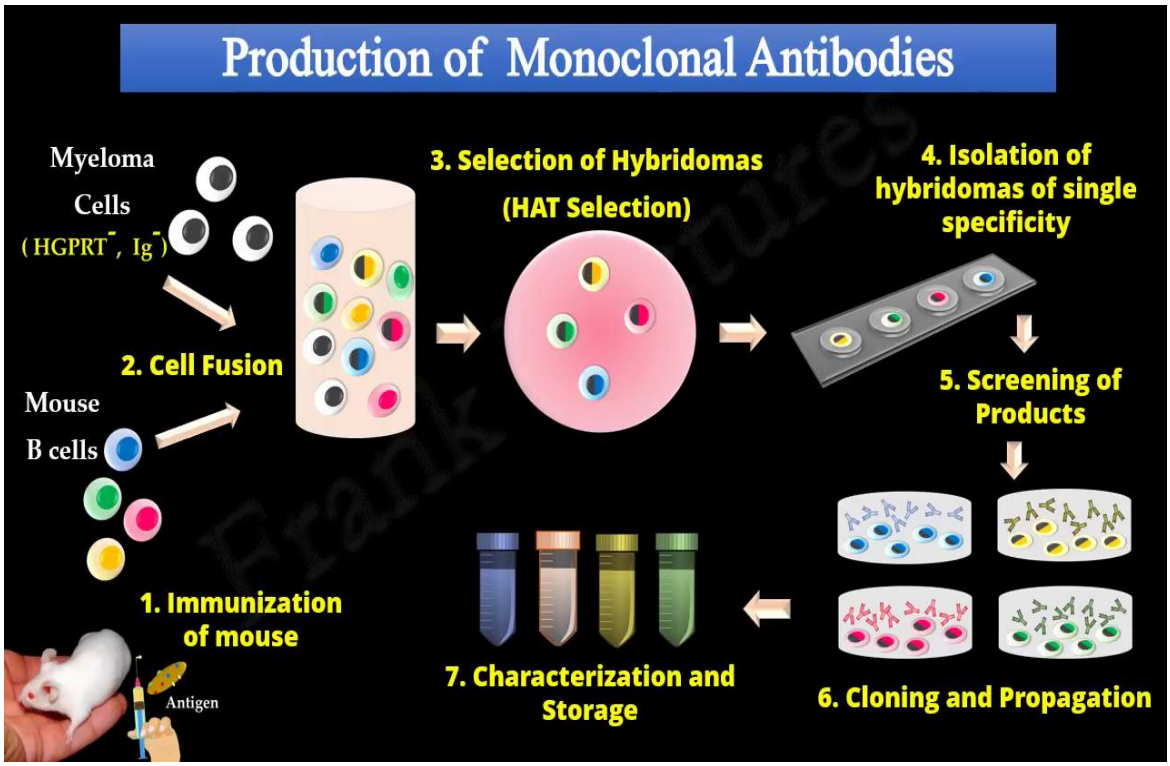
Then the dilution of the incubated medium is carried out in 96-well plates. The B-cells produce the antibodies and are directed towards the same epitope, and are thus monoclonal antibodies

5.SCREENING TECHNIQUE

The next stage is the identification and selection of
hybridomas which produce specific antibodies. This is done by ELISA technique in which the hybridoma culture supernatant, secondary enzyme labeled conjugate, and chromogenic substrate are incubated with the
formation of a colored product indicates a positive hybridoma.

6.CLONNING AND PROPAGATION

Many identical daughter clones can also be produced from the B cell that produces the desired antibodies.Thehybridoma colony is then subjected to grow in culture medium thereby producing the antibodies.Hybridomas are grown in multiwell plates and then followed by their growth in larger tissue culture flasks.



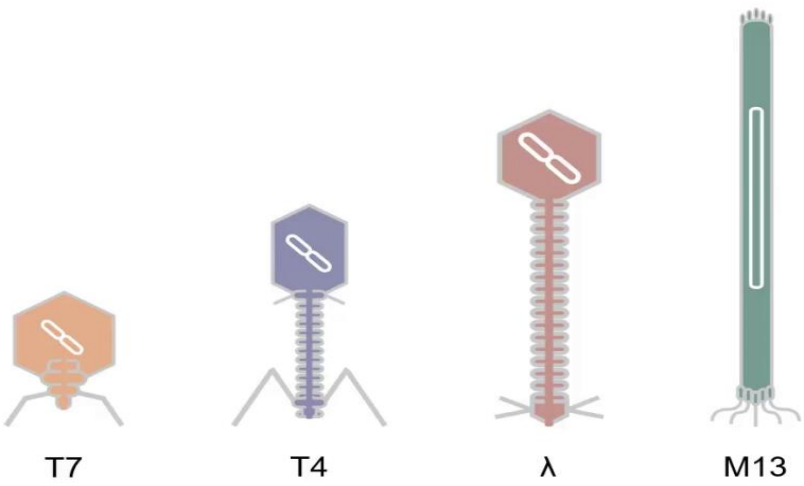
Although mouse hybridoma technology has pioneered the production of mAbs, and it remains an important platform both in research and industry, this method is time-consuming and restricted to particular animal species. Low fusion efficiencyof a B cell and myeloma partner also limits the chance of recovery of desired mAb clones. Technologies such as phage, yeast, and mammalian cell display have been widely recognised as powerful methods that increase the variety of mAbs screened from immunised or synthetic repertoires. However, in most display systems, the parental libraries are constructed through random combination of heavy and light chain genes.

B.PHAGE DISPLAY TECHNOLOGY

Phage display technology was developed by **G. Smith in 1985** to display the peptides on the surface of lysogenic filamentous bacteriophages.

General view to biology of filamentous phages

Filamentous phages are group of non-lytic phages that incorporate round single stranded DNA. The family of Ff, M13,fd, and f1 are vital phages which have utility in phage display.M13 phage is the most generally used.The most important feature is that in contrast to other phages, M13 can be effortlessly purified and used.



METHOD

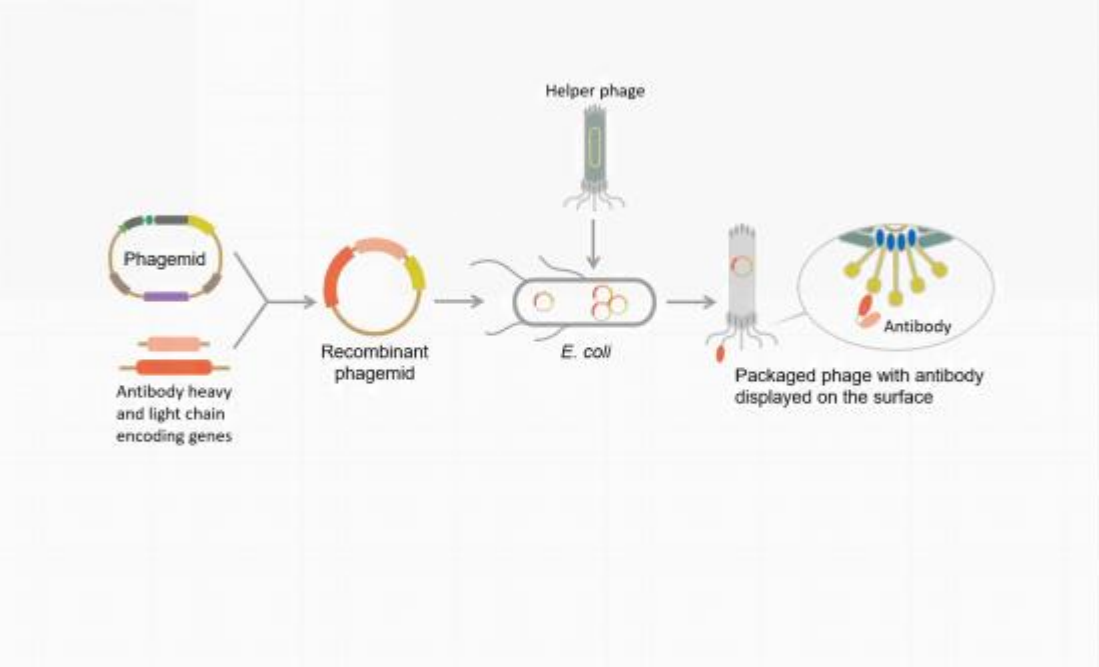
Production of gene fragment:

This phase involves animal immunization with the desired antigen and then isolation of B lymphocytes, mRNA extraction and cDNA synthesis.

Cloning of gene fragments in the phagemid vectors:

Genes related to the different clones of antibodies are digested with restriction enzymes, clone into phagemid vectors and then display on the surface of phages.These vectors help to the displayed antibodies to maintain their function at the surface of phage. But using of

phages like M13 that does not destroy the bacteria cells is most applicable.

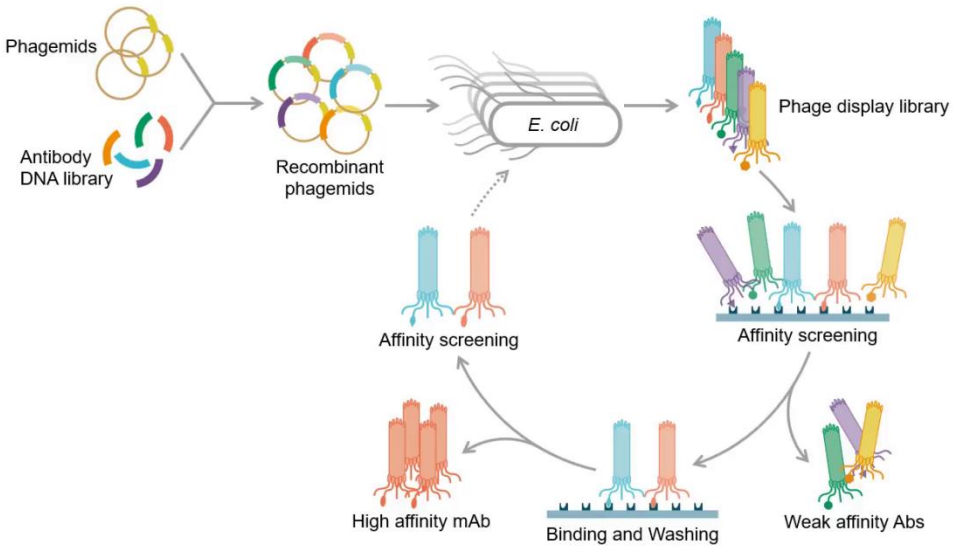


Selection of specifific phages:

After cloning of the fragmentsinto phagemid vectors, because of diversity in antibody gene,variety of clones of antibodies display on the surface of the phage. Selections of specific clone thatrecognize the antigen(target of interest) perform by biopanning. At this step expressed antibodies on the surface of phage based on their ability to bind to the target antigen will be enrichedthroughbiopanning. The panning processesincludeimmobilization of antigen, binding of the phages,washing and removing non bonded phages,elution of the bonded phages, re-infection of the bacterial cells to amplify the eluted phages, purification of the recombinant phages and re-expression of antibodies on the surface of phage. Biopanning usually repeats 3 to 5 times to isolate specific antibodies with high affinity to target.

Screening:

Isolation of antibodies with high affinity to target is the main aim of this step. Screening is performed using different methods like: immunoassay,immunocytochemistryactive isolation of cells due to their fluorescent properties and immunoblotting.



C.ECOBODY TECHNOLOGY

A rapid and cost-effective monoclonal antibody screening method from single animal B cells using reverse transcription (RT)-PCR and Escherichia coli cell-free protein synthesis (CFPS), which allows evaluation of antibodies within 2 days.

Method

Antigens

Bacteria

V. parahaemolyticus

E. Coli

These are cultured in Luria-Bertani (LB) medium at 37 °C overnight, and inactivated by incubation at 80 °C for 30min in phosphate-bufered saline (PBS) containing 0.25% formalin.

Immunization of ANIMAL MODEL

Model used were immunised with inactivated bacterial cells by hypodermic injection. The second and third boosters were given after intervals of 2 weeks and 10 days, respectively. Blood samples were collected 2 days
afterthefinal booster.

Selection of B cells and antigen-coated magnetic beads

To select B cells producing mAbs, 1 µM ER-Tracker is used.The cells with higher fuorescence intensity were then sorted.

Amplification of mAb genes from single B cells

The following three steps were performed without a break:

i. Reverse transcription (RT).

ii. First PCR. Immediately after RT was finished, 0.5 µL of reaction mixture was used as the template for this PCR with gene specific primers for Lc and Hc, separately.

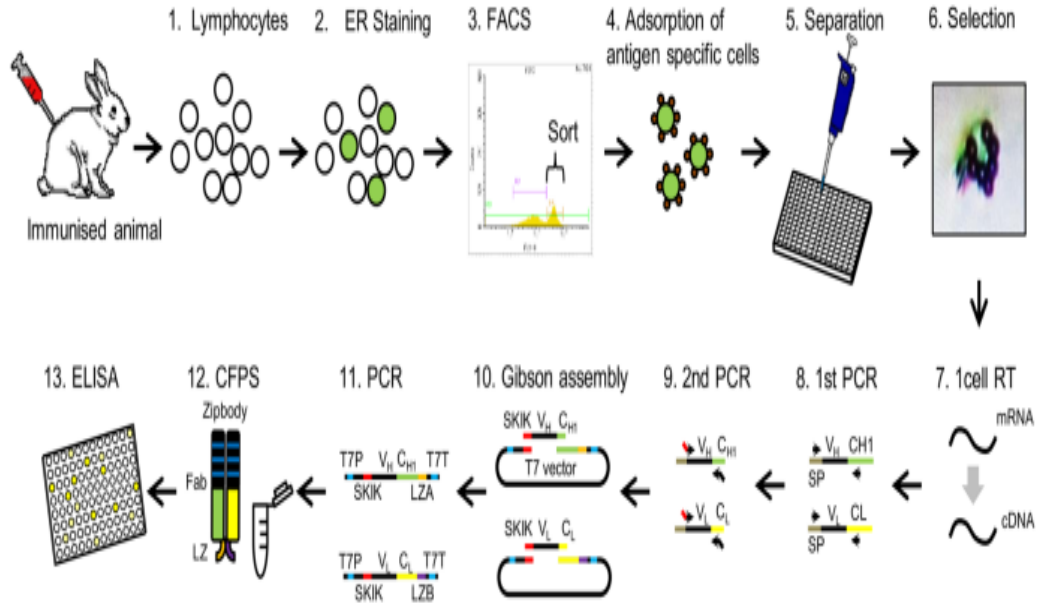
iii Second PCR

Construction of DNA fragments for CFPS. Continuously, from the previous DNA amplification steps,

we prepare DNA fragments for CFPS and plasmids for transformation.

ELISA.
Unless otherwise stated, mAbs produced in CFPS or E. coliwere analysed by SDS-PAGE in reducing conditions and
ELISA using our standard protocol.

DIAGRAMATIC PRESENTATION OF ECOBODY TEHNOLOGY



7.APPLICATIONS OF MONOCLONAL ANTIBODIES

Clinical application of monoclonal antibodies include
A.Diagnostic applications
B.Therapeutic application
C.Drug Delievery

A. Diagnostic applications

Monoclonal antibodies are utilized in diagnostic kits for the diagnosis of various
1.Infectious diseases,
2.Monitoring drug levels
3.Detecting pregnancies
4.Matching histocompatibility antigen detecting
5.Diabetes, cancer and in immunoscintigraphy
Radiolabeled MAB are used in diagnostic purpose. The technique is called the immunoscintigraphy. In this technique a planer gamma camera is used to detect the distribution of gamma entitling radioisotopes conjugated with MAB in a two dimensional manner e.g. Technetium Tc-99m nofetumomab merpentan (for lung cancer diagnosis).

1. CVS Diseases

Myoscint(For Myocardial Infraction) is the first MAB based imaging agent in market in much European

country. The product consists of a kit containing 0.5 mg of antimyocin fab fragment conjugate with chelator DTPA (diethyl triamine penta acetic acid) this is labeled by mixing with (indium chloride) after incubation of 10 minute.
Radio labelled 99mTc-Y22 is used for thrombus imaging in vivo as it act as antifebrin.

2. Infectious diseases

mAb are important diagnostic reagents used in biomedical research, microbiological research in diagnosis of Hepatitis, AIDs, influenza, herpes simplex, Chlamydia infections.
For Hepatitis C mAb (7G9) bind with antigen HCV E1/E2 and detected by ELISA.
Anti HIV-1 p24 diagnose the presence of HIV infection.

3. Cancer

MABs are being evaluated for detection of different type of cancer like breast carcinoma, ovarian carcinoma, and lung carcinoma. These MABs can be targeted against many type of tumor.
EXAMPLES
1 Humaspect (Votumumab) Detection of colon or rectal carcinoma
2 Tecnemab KI (murine Mab fragments) Diagnosis of cutaneous melanoma lesions
3 OncoScint CR/OV (Satumomab) Detection/staging/follow up of colorectal and ovarian cancers

4. Detecting pregnancy

Home pregnancy tests can find the presence of a pregnancy hormone HCG in a sample of urine.
HCG level increases quickly. After conception level of HCG increases in body that appears in urine too, urine is collected and placed on test zone, antibodies are present there that grasp HCG which is in contact with dye activating enzyme, activated by complex and produces a sharp red color that proves that the result is positive.

B. Therapeutic applications

Few examples are given such as

1. Anti-inflammatory

1 Remicade (Infliximab) Treatment of Crohn s Disease
2 Humira (adalimumab;anti-TNF) Rheumatoid arithritis
3 Xolair (omalizumab) Anti IgE Antibody antagonize IgE Receptors on mast cells Persistant asthma

2. Immunosuppressants

1 Zenapax (Daclizumab) IL2 Antagonist Prevention from acute kidney transplant rejection
2 Simulect (Basiliximab) Prophylaxis in acute transplant rejection

3. CVS Disorders

1 ReoPro (Abciximab) directed against platelets surface receptors,Prevention from blood clot

4. Anti-cancer

These are classified in further classes

a. Cytotoxic Monoclonal antibody mediated therapy recruits cytotoxic cells (monocytes and macrophages) through antibody-dependent cell cytotoxicity

Alemtuzumab (Campath®): for chronic lymphocytic leukemia.

b. Chemolabelled

These mAbs have powerful chemotherapy (or other) drugs attached to them. They are also known as antibody-drug conjugates (ADCs). (The drug is often too powerful to be used on its own – it would cause too many side effects if not attached to an antibody.)

1. Brentuximab vedotin (Adcetris®): Antibody is attached to drug named MMAE. This drug is used to treat Hodgkin lymphoma and anaplastic large cell lymphoma.
2. Ado-trastuzumab emtansine: It's used to treat breast cancer.

c. Radio labelled

Radiolabeled antibodies: Radiolabeled antibodies have small radioactive particles attached to them. Ibritumomab-tiuxetan (Zevalin®) is an example of a radiolabeled mAb. The antibody delivers radioactivity directly to cancerous B cells and can be used to treat some types of non-Hodgkin lymphoma.

5. Anti-infectious

1 Palivizumab Treating infection caused by respiratory Syncytial virus
2 Ibalizumab It cause conformational change in CD4 receptor proteins on T cells so HIV can't enter in cell

6. Monoclonal antibodies for drug delivery

MoAb and radioimmunoconjugates, there has been some success in targeting toxins (i.e., immunotoxins) and drugs (i.e.,drug-immunoconjugates) using MoAbs as targeting agents.

a. Toxin conjugates: several toxin proteins like diphtheria toxin and ricin have been conjugated to tumor specific antibodies, with moderate to high degree of success in tumor drug delivery.

b. Immunoliposomes: The antigens expressed by tumor cells are not specific but are merely present in higher ratio than on the normal cells. Hence, systems such as immunoliposomes have been developed to exploit these opportunities, as they are expected to bind to a greater extent to high antigen density tumor cells than to low antigen density normal cells. Methotrexate-g-aspartate can be conjugated to either specific (anti-K2Kk IgG2A).

c. Immunomicrospheres: The microspheres bearing Lewis lung carcinoma MoAbs demonstrated slightly higher localization in lung carcinoma at 24 h after its administration. Recent approach combines MoAb targeting with enzymatic prodrug activation. In this therapeutic method, called antibody-directed prodrug therapy (ADEPT), an enzyme–antibody conjugate is administered and allowed to accumulate in the target site (e.g., tumor). A latent, non-toxic prodrug is then injected, which on contact with the enzyme is converted into the active parent drug and subsequently kills the tumor cells. For example, a glutamic acid derivative of benzoic acid mustard was administered to choriocarcinoma-bearing mice, followed by a carboxypeptidase-antibody conjugate that cleaved glutamic acid from the active drug.

8. ROUTES OF ADMINISTRATION OF MONOCLONAL ANTIBODIES

Appropriate route of administration is driven by
Efficacy
Safety
Cost effectiveness
Patient preference
Which ensure optimal treatment adherence Mostly 2 routes are available for monoclonal antibody administration
1. I.V
2. Subcutaneous

1. Intravenous

mAbs are often administered by the IV route, which provides maximum bioavailability with minimal risk of immunogenicity.
IV administration is suitable for medications that are administered in large volumes and those that may cause irritation.

The drawbacks of IV administration include the fact that it often requires dedicated personnel, which likely makes it less convenient and, if performed at a hospital, more expensive.

2. Subcutaneous

SC administration is less invasive than IV administration. It may be performed by the patient and therefore, is associated with shorter clinic visits and more optimized use of healthcare resources.

Disadvantages of SC administration include slower absorption and lower bioavailability than IV, as well as higher probability of injection-site reactions and pain associated with administration of large fluid volumes.

In an observational study of 201 patients with rheumatoid arthritis who were receiving treatment with IV infusions when asked if they would switch to SC administration, patients (54.2%) who chose to switch from IV to SC administration found SC injections more convenient than continuing IV therapy.

Animal models of lung cancer to test aerosol delivery of cetuximab (an approved anti epidermal growth factor receptor antibody) is also under consideration.

9. CHALLENGES AND FUTURE PROSPECTS

A. Antibody related.

While most mAbs can display prolonged circulation times by FcRn-mediated recycling, e,g bevacizumab

have life of 7-10days can increase the chances of undesirable side effects.

mAbs are limited in their ability to penetrate and accumulate in tissues due to their large size.

Subcutaneous administration is a desired route of administration, macromolecules are likely to be restricted.

Enzymatic degradation.

B. Formulation related.

Protein folding due to functional groups

Tertiary structure of biologics are susceptible to physical stress from the environment causing aggregation

10.MERITS AND DEMERITS

Sr. #	Merits	Demerits
1	Different clones of antibodies can be generated to different epitopes on a single antigen.	Production of monoclonal antibodies is more labor-intensive.
2	Hybridoma cells can serve as an infinite source of the same antibody.	They may be limited in their applications.
3	their homogeneity is very high and they provide consistent, reproducible results.	A vast majority of monoclonal antibodies are produced in mice because of a robust myeloma cell line.
4	They bind only to one antigen in a mixture of related proteins.	High specificity of monoclonal antibodies limits their use in multiple species.
5	Batch–to-batch variability is very minimal.	----------