

A Comprehensive overview of monoclonal antibodies production through various methods and applications.

¹Daphne sherine.S

Associate professor

Department of pharmaceutics

Psv.college of pharmaceutical science and research, Krishnagiri, 63,phase 4, upkar royal garden zuzuvadi,hosur 635126.INDIA

²Thirumalai.P, ³Poovarasana.P, ⁴Suhasini.G, ⁵Thilagavathi.A, ⁶Raghul.R,

UG Students

Psv. college of pharmaceutical science and research, Krishnagiri.

Abstract- In biomedical research, monoclonal antibodies are an indispensable tool. In 1975, Cesar Milstein and Georges J.F. Kohler developed the process for making monoclonal antibodies, for which they were awarded the 1984 Nobel Prize. Antigen injection to mice results in an immunological response, which is used to create monoclonal antibodies. The injected mouse is subsequently collected for its B cells. The utilisation of transgenic mice, the phage display platform, the hybridoma technologies, and the production of monoclonal antibodies from B-cells are all introduced in this overview. The types of mAbs created include humanised mAbs, which contain 10% mouse protein, chimeric mAbs, which contain 25% mouse protein, and murine mAbs, which contain 100% mouse protein. mAbs are utilised for cancer treatment, cancer diagnosis, and transplant rejection prevention. The synthesis of monoclonal antibodies from plants and the use of transgenic mice are both emerging strategies, but hybridoma technology is a more conventional approach. Polyclonal antibodies are not desired; monoclonal antibodies are. In comparison to hybridoma approach, the phage display method is a sophisticated technology that can be relied upon to create mAb at a faster rate. In-vitro and in-vivo procedures are used to continue both the hybridoma technique and the phage display method. Plastic cell culture equipment is required for the cell suspension culture technology, which can be applied to both small and big groups of people. The market value of mAbs in the medical industry is anticipated to increase significantly in the future.

Key words: Monoclonal antibodies; Hybridoma technology; Phage display method; Cancers; Graft rejections

1. INTRODUCTION

1.1 ANTIBODIES

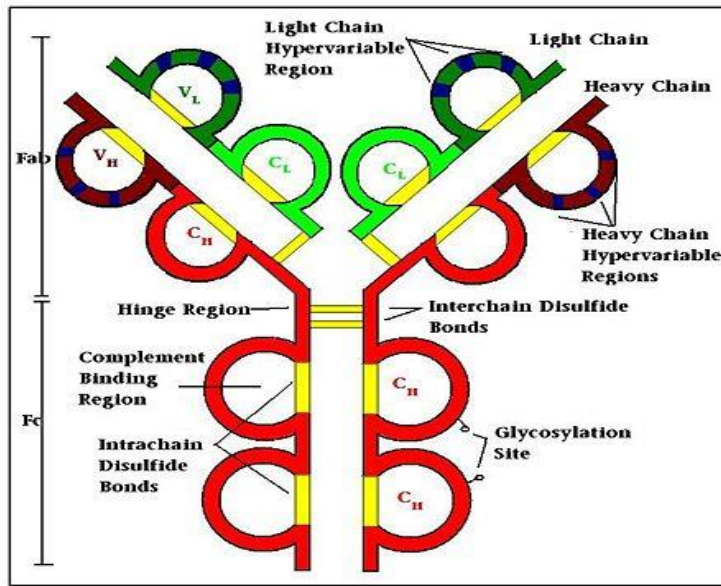
Activated B cells (plasma cells) create antibodies, also known as immunoglobulin, which is a specialised glycoprotein that can combine with the antigen that caused it to be produced. (1)



[Fig.01-Antibody]

➤ It is a large, Y-shaped protein that the immune system employs to recognise and destroy alien substances like harmful bacteria and viruses. (2,3)

1.2 STRUCTURE



[Fig.02-Ig structure]

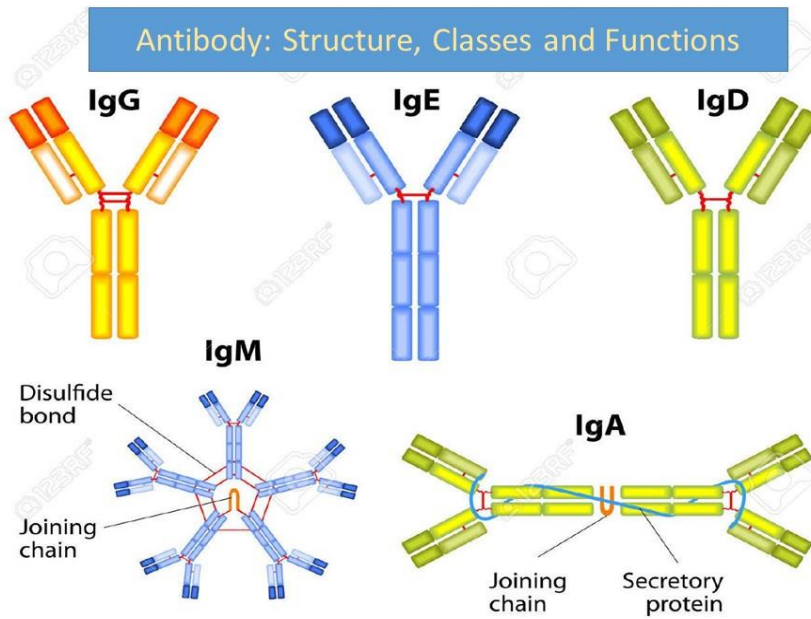
A sizable Y-shaped structure is an antibody. It recognises the pathogen's antigen-containing segment. There are two heavy chains and two light chains in it. The disulfide bonds that connect them. Both light chains and heavy chains have two regions that are constant and two regions that are variable. Light chains are referred to as kappa or lambda chains depending on their structure and amino acid sequence. But gamma, alpha, muta delta, and epsilon are used to identify heavy chains. Heavy chains feature a flexible hinge region in the centre

- Each light and heavy chain has two terminal ends: a N terminal, also known as an amino terminal end, and a C terminal, also known as a carboxyl terminal end. By a single interchain disulfide connection, each light chain is connected to the heavy chain as well. However, two disulfide bonds connect the two heavy chains together. Also present are Fab [fragment antigen binding] FC proteins.
- There are two Fab regions at the top of the Y. While Fab fragments contain both constant and variable areas, FC fragments only contain constant regions.⁽⁴⁾

1.3 .TYPES OF ANTIBODIES

Type of antibody	Function
IgA	Found in body secretions like breast milk and saliva, and prevent antigens crossing epithelial membranes and invading deeper tissues
IgD	This is made by B-cells and displayed on their surfaces. Antigens bind here to activate B-cells
IgE	Found on cell membranes of, e.g., basophils and mast cells, and if it binds its antigen, activates the inflammatory response. This antibody is often found in excess in allergy
IgG	This is the largest and most common antibody type. It attacks many different pathogens, and crosses the placenta to protect the fetus
IgM	Produced in large quantities in the primary response and is a potent activator of complement

[Table 01: Types of Ig

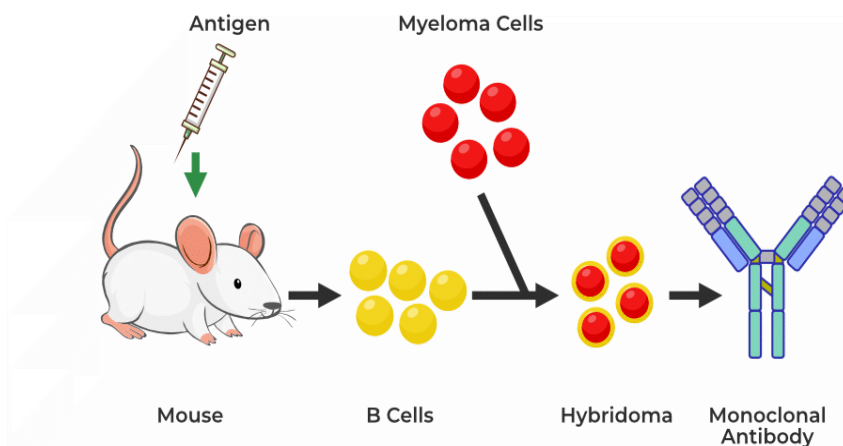


[Fig.03-Types of Ig structure]

1.4. MONOCLONAL ANTIBODIES

Monoclonal antibodies are made from protein molecules generated from hybridoma cells by recombinant DNA technology. (6)

➤ Monoclonal antibodies can be produced using either live animals or cell culture. When hybridoma cells are injected into the peritoneal cavity of mice, the cells grow into tumours that release ascites fluid, a fluid that is brimming with antibodies.



[Fig.04-mAb]

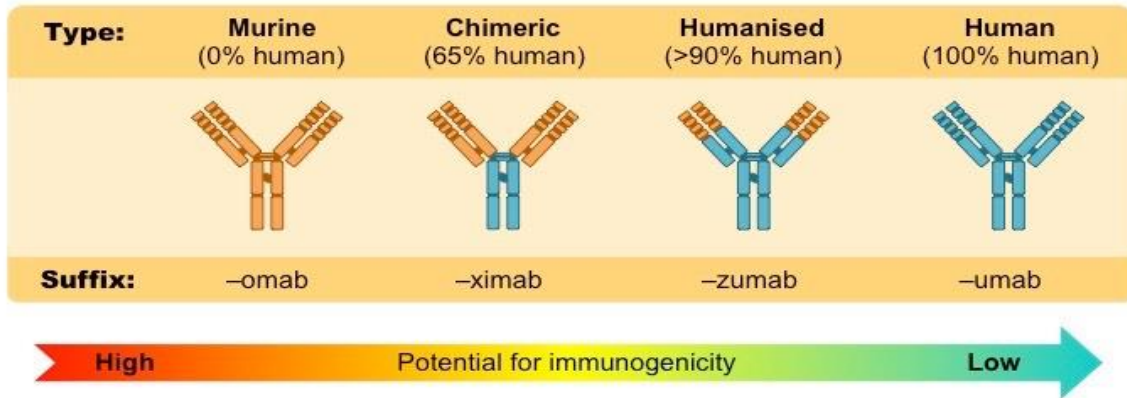
Cell culture-based fermentation chambers enable large-scale production. When administered therapeutically, a particular monoclonal antibody is referred to as a mAb. (7) Monospecific antibodies (or "mAbs") are those that are made by immune cells that are all exact replicas of a single parent cell.

➤ mAbs are superior than polyclonal antibodies in terms of their controlled manufacturing procedures and their repeatable affinity for specific target antigens. mAbs are crucial for guarding against food poisoning, preserving health, and investigating environmental toxins. Despite their widespread use and significance, the majority of people have never heard of mAbs or how they have transformed healthcare and spawned a completely new industry. The billions of tiny antibodies that our immune systems manufacture every day to combat substances called antigens that are deemed to be foreign or potentially dangerous are used to make mAbs in laboratories. The blood of mammals, including humans, contains countless varieties of antibodies. Each antibody is extremely selective, which means it can only bind to one particular antigen. Antigens can be live things like bacteria, viruses, fungus, parasites, pollen, or nonliving things like toxins, chemicals, medications, or foreign particles that are thought to be foreign to the body. B lymphocytes, a type of white blood cell, generate antibodies. When antibodies have located their antigen, they might attack it along with other immune system-produced cell types. (6)

1.5 CHARACTERISTICS OF mAb

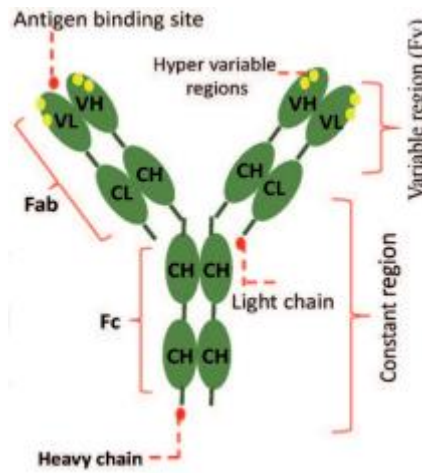
- ✚ Only one class of antibodies are produced; it only contains one antibody that can recognise one determinant.
- ✚ It only generates one type of antibody.
- ✚ A particular antibody can be produced utilising an impure antigen.
- ✚ It can be replicated very well. (8)

1.6 TYPES OF mAb



[Fig.05-Types of mAbs]

A. Murine mAbs:



[Fig.06-Murine mAb]

The term "murine mAbs" refers to the antibodies made by murine hybridomas, which are created when lymphocytes and murine myeloma cells fuse. Murine mAb is no longer widely used in therapeutic settings. Murine mAb use has some minor side effects, such as cytotoxicity stimulation, but prolonged use might result in serious symptoms, such as anaphylactic shock and allergies. The formation of human anti-murine antibodies against murine immunoglobulin (HAMA response), which follows from the introduction of these murine antibodies to a person, renders the mAb ineffective since it induces allergic and unpleasant reactions. The mouse immunogenic components that trigger an immune response were eliminated to prevent undesired reactions in the body. Despite this, the first approved mAb for therapeutic use in humans was OKT-3, an anti-CD3 mAb derived from mouse hybridoma cells. Other instances: 131I-Tositumab with 90Y-ibritumomab.^(9,10,11)

[Table 02: List of murine mAbs in use and their year of FDA approval]

Name of MAb	Type of MAb	Therapeutic use	Year of FDA approval
Imciromab penlelele	Murine MAb	Detection myocardial injury	1996
Arcitumomab	Murine MAb	Diagnosis	1996
Orthoclone	Murine MAb	Immunological use	1992
FanolesomaB technetium Tc 99m	Murine MAb	Diagnosis	2004
Nofetumomab	Murine MAb	Diagnosis	1996
Ibritumomab tiuxetan	Murine MAb	Cancer	2002
Capromab pentitide	Murine MAb	Cancer	1996

B. Chimeric mAbs:



[Fig.07-Chimeric mAb]

"Chimeric mAbs" refers to therapeutic antibodies created by fusing human and non-human genetic material, where the non-human genetic material may be taken from mice or other non-human animals. The chimeric mAb is made by replacing the constant section of mouse immunoglobulin with a constant piece of human immunoglobulin. It is sometimes referred to as a mixture of a constant region of human immunoglobulin with a variable region of mouse immunoglobulin. Despite some unfavourable immune responses seen after chimeric mAbs were given to humans, these antibodies still contain roughly 65% human genetic material and 30% foreign DNA. Such chimeric mAbs as Infliximab, Rituximab, and Abciximab have received FDA approval.^(9,10,11)

[Table 03: List of chimeric mAbs in use and their year of FDA approval]

Name of MAb	Type of MAb	Therapeutic use	Year of FDA approval
Brentuximab vedotin	Chimeric MAb	Cancer	2011
cetuximab	Chimeric MAb	Cancer	2004
infliximab	Chimeric MAb	Immunological use	1998
abciximab	Chimeric MAb	hemostasis	1993
rituximab	Chimeric MAb	Cancer	1997
basiliximab	Chimeric MAb	Immunological use	1998
siltuximab	Chimeric MAb	Immunological use	2014
dinutuximab	Chimeric MAb	Cancer	2015

C. Humanized mAbs:



[Fig.08-Humanised mAb]

In order to prevent the undesirable immune reactions when introduced into humans, humanised mAbs are created by humanising the non-human variable section of antibodies. Here, the non-human genetic component has been cut down to between 5% and 10%, making up roughly 95% of the composition. Using a chain-shuffling randomization approach, the CDRs from mice mAb are transferred to human IgG to complete the humanization process. Daclizumab, omalizumab, and alemtuzumab are three humanised monoclonal antibodies that have been given FDA approval.^(9,10,11)

[Table 04: List of humanized mAbs in use and their year of FDA approval]

Name of MAb	Type of MAb	Therapeutic use	Year of FDA approval
tocilizumab	Humanized MAb	Immunological use	2010
bevacizumab	Humanized MAb	cancer	2004
alemtuzumab	Humanized MAb	Immunological use	2001
certolizumab	Humanized MAb	Immunological use	2008
trastuzumab	Humanized MAb	cancer	1998
elotuzumab	Humanized MAb	cancer	2015
vedolizumab	Humanized MAb	Immunological use	2014
obinutuzumab	Humanized MAb	cancer	2013
Ado-trastuzumab emtansine	Humanized MAb	cancer	2013
Pembrolizumab	Humanized MAb	Cancer	2014
Alemtuzumab	Humanized MAb	Immunological use	2001
Ranibizumab	Humanized MAb	Ophthalmic use	2006
Gemtuzumab ozogamicin	Humanized MAb	cancer	2000
mepolizumab	Humanized MAb	Immunological use	2015
pertuzumab	Humanized MAb	cancer	2012
idarcizumab	Humanized MAb	hemostasis	2015
efalizumab	Humanized MAb	Immunological use	2003
eculizumab	Humanized MAb	hemostasis	2007
palivizumab	Humanized MAb	Antiviral	1998

D. Fully human mAbs:

[Fig.09-Fully human mAb]

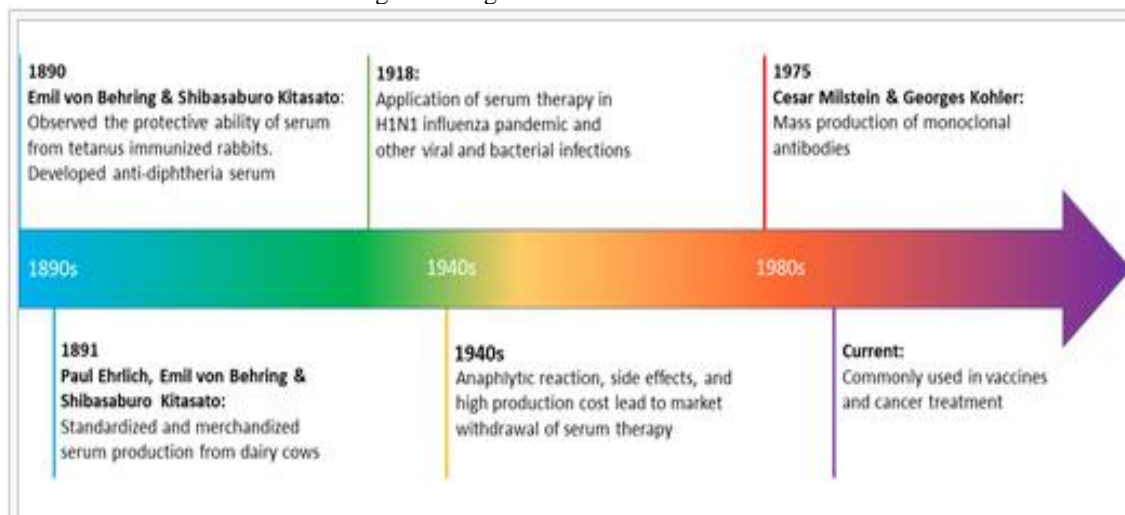
When utilised for therapeutic purposes by expressing only human genes, the human mAb is produced using human hybridomas by phage display or Tran's chromosome mice technique in order to prevent the unfavourable immune reactions. Here, immunisation with transgenic mice can produce a hybridoma that produces human antibodies. Since this human mAb contains solely human genes, it is thought to be a safe medication for people. However, this approach is challenging since the cell lines that were cultivated should have immortality, yet human mAbs were created as a substitute for murine mAbs. Humira, a fully human mAb, was initially identified in 2003 to treat rheumatoid arthritis. Adalimumab and panitimumab are two further mAbs that have been given approval, although there are still some human mAbs that are undergoing clinical trials.^(9,10,11)

[Table 4: List of human mAbs in use and their year of FDA approval]

Name of MAb	Type of MAb	Therapeutic use	Year of FDA approval
Ofatumumab	Human MAb	Cancer	2009
Belimumab	Human MAb	Immunological use	2011
Secukinumab	Human MAb	Immunological use	2015
Ramucirumab	Human MAb	Cancer	2014
Daratumumab	Human MAb	Cancer	2015
Adalimumab	Human MAb	Immunological use	2002
Canakinumab	Human MAb	Immunological use, anti-inflammatory	2009
Nivolumab	Human MAb	Cancer	2014
Necitumumab	Human MAb	Cancer	2015
Alirocumab	Human MAb	Lipid-lowering	2015
Denosumab	Human MAb	Bone disorders	2010
Raxibacumab	Human MAb	Anti-toxin	2012
Evolocumab	Human MAb	Lipid-lowering	2015
Golimumab	Human MAb	Immunological use	2009
Ustekinumab	Human MAb	Immunological use	2009
Panitumumab	Human MAb	Cancer	2006
Denosumab	Human MAb	Cancer	2010
Ipilimumab	Human MAb	Cancer	2011

1.7 HISTORY

It is not a novel concept to use a patient's recovered blood to aid in the recovery of another patient who is suffering from the same ailment. In order to stop the spread of the illness during the 1918 influenza virus pandemic, doctors gave those who had recovered from the illness blood serum to give to those who were already afflicted. It was thought that the immune system had produced antibodies in this blood to recognise and get rid of infections.



[Fig.10-History and development of mAbs]



Niels K. Jerne



Georges J.F. Köhler



César Milstein

[Fig.11-Scientists behind the discovery of mAbs]

➤ Georges Kohler of West Germany and Cesar Milstein of Argentina discovered hybridoma technology in 1975, and they shared the 1984 Nobel Prize in Physiology or Medicine with Niels Kaj Jerne of Denmark. In 1976, Kohler and Milstein created a method to combine tumorous myeloma cells with splenocytes, which were taken out of the spleen of a mouse that had received an immunisation. The hybrid cells are quick to multiply and create very large quantities of antibody because they are clones of cells that manufacture antibodies against a desired antigen. The hybridoma can preserve the antibody genes of mouse spleen cells due to its capacity for rapid proliferation and high antibody secretion rates, similar to those found in myeloma cells. In order to prevent the reactions and responses seen in patients given injections of murine-derived mAbs, Greg Winter utilised the first humanised MAbs in 1988.

➤ Numerous infectious disorders, including Lassa fever, hemorrhagic fever, measles, and different respiratory viruses, have been treated this way throughout the past century. We can isolate and produce particular antibodies to combat a range of diseases thanks to technological progress over the past 30 years. Monoclonal antibodies are the name given to these particular antibodies. Over 30 monoclonal antibodies have currently been licenced for use in medicine to treat a variety of illnesses.^(12,13)

3.VARIOUS PRODUCTION METHODS

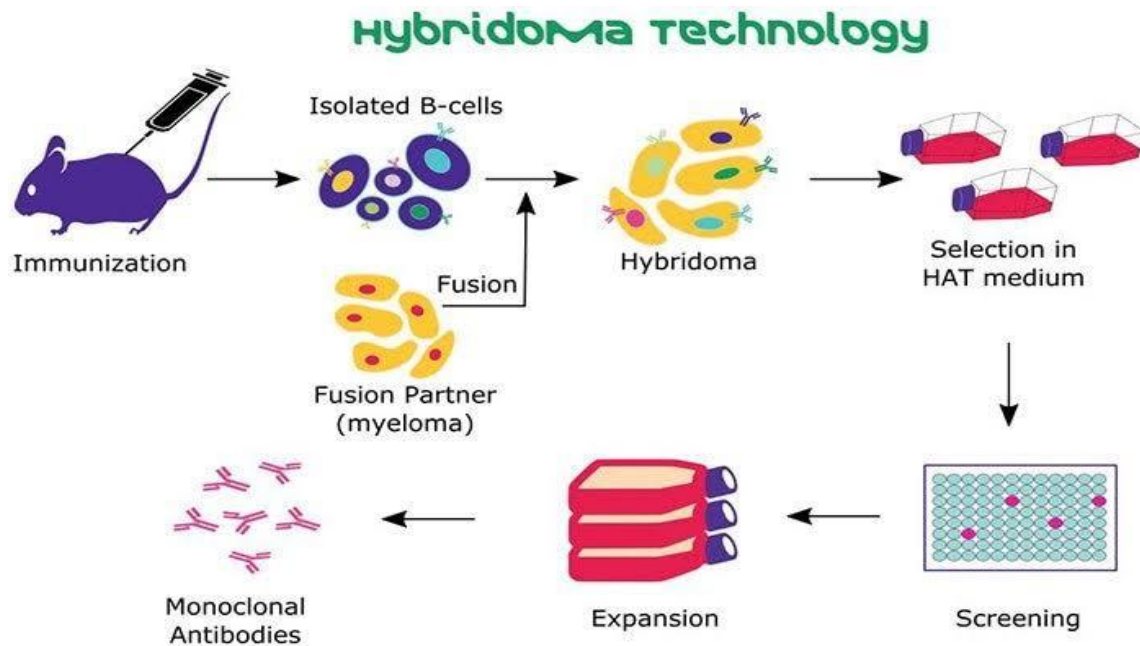
Even though just a limited amount of mAb needs to be manufactured for laboratory research purposes, it needs to be produced on a big scale because it is utilised as a medicinal treatment. There are numerous other mAb production methods that are evolving, and many additional alternative methods are also emerging and being found. A prescription medication mAb is manufactured on a large scale utilising adherent or suspension cells, and some effective techniques are used to ensure a better cell culture. In contrast, the method using suspension cells is thought to be simpler than previous methods.

3.1.Hybridoma Technology:

In vitro hybridization of myeloma cells with activated B lymphocytes is referred to as a hybridoma. Hybridoma technology is the process of using hybrid cells to produce monoclonal antibodies.⁽¹⁴⁾

Steps involved in hybridoma technology:

- 1.1. Immunization
- 1.2. Cell fusion
- 1.3. Selection of hybridomas
- 1.4. Screening the products
- 1.5. Cloning and propagation
- 1.6. Characterization and storage



3.1.1. Immunization:

In hybridoma technology, immunising an animal (often a mouse) with the proper antigen is the initial step. The antigen is administered subcutaneously combined with an adjuvant, such as Freund's complete or incomplete adjuvant (adjuvants or not particular potentiators of certain immune responses). Several injection sites or multiple repetitions. This permits B-lymphocytes that are reacting to the antigen to be stimulated more frequently. A final dosage of antigen is injected into the animal three days before it is killed. By using this method, the immune-stimulated cells that synthesise antibodies have developed the fastest. The animal's serum concentration at regular intervals throughout the vaccination process.

The animal has been killed once the serum antibody concentration is at its ideal level. To release the cells, the spleen is surgically or enzymatically ruptured and aseptically removed. By using a density gradient centrifuge, the lymphocytes in the spleen are separated from the other cells.

3.1.2. Cell fusion:

The myeloma cells with HGPRT defects are combined with the completely cleansed lymphocytes. Since polyethylene glycol (PEG) is poisonous, the mixture of cells is only exposed to it for a brief time (a few minutes). Cells are retained in a fresh medium once PEG is washed away. These cells are a mix of free lymphocytes, free myeloma cells, and hybridomas (fused cells).

3.1.3. Selection of hybridomas:

Only the hybridoma cells proliferate when the cells are cultivated in HAT media (according to the above-mentioned concept), while the other cells gradually die off. In 7–10 days of culture, this occurs. It is crucial to choose a single set of hybrid cells that produce antibodies. If the hybridomas are separated and developed separately, this is feasible. Because the hybridoma cells are suspended in such a thin solution, each aliquot typically contains one cell. When these cells are cultured in a typical culture medium, the desired antibody is produced.

3.1.4. Screening the products:

The hybridomas need to be examined for the desired specificity of the antibody secreted. Each hybridoma culture's grown cells are periodically evaluated for the necessary antibody specificity. The two methods, ELISA and RIA, are frequently employed for this. Both assays require the antibody to attach to a particular antigen, which is typically coated on plastic plates. The unbound antibody and other elements of the medium may then be removed, and the hybridoma cells that are making the necessary antibody can then be found through screening. Monoclonal antibody refers to the antibody released by hybrid cells.

3.1.5. Cloning and propagation:

The isolated and cloned single hybrid cells that are producing the required antibodies. The limiting dilution method and the soft agar method are the two procedures frequently used for cloning hybrid cells.

3.1.5. A. Limiting dilution method:

In this process, aliquots from each successive dilution of the hybridoma cell suspension are added to microculture wells. Each aliquot in a well of the dilutions only includes a single hybrid cell. By doing this, the production of monoclonal antibodies is confirmed.

3.1.5. B. Soft agar method:

The hybridoma cells are cultivated on soft agar using this method. Colonies can be formed by the simultaneous growth of many cells in a semisolid medium. These colonies will be monoclonal in nature. In reality, the two aforementioned methods are combined and applied to produce mAbs at their highest rate.

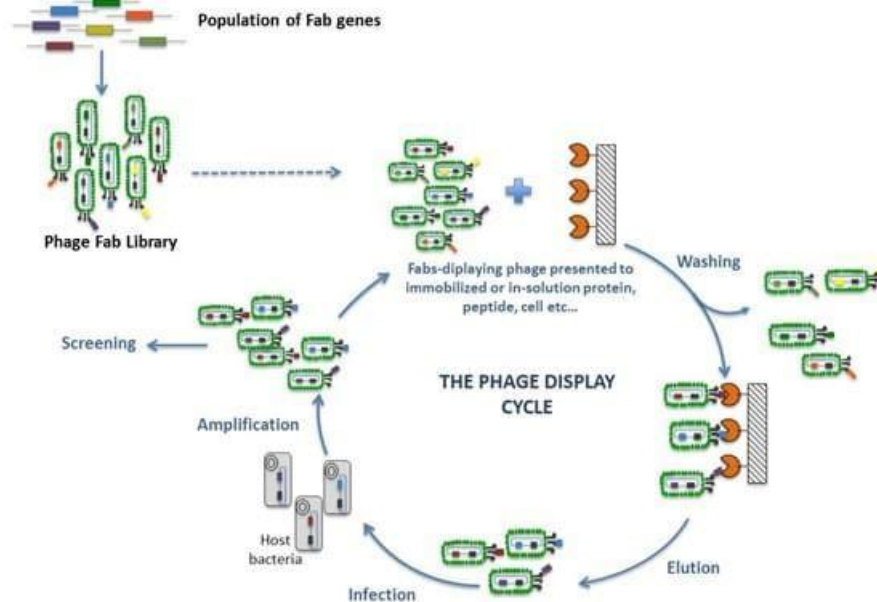
3.1.6. Characterization and storage:

The biochemical and biophysical characterization of the monoclonal antibody is necessary to determine whether it has the requisite specificity. It's crucial to identify the mAbs immunoglobulin class or subclass, the epitope it is specific for, and the number of binding sites it has. It's crucial that the mAbs and cell lines remain stable. The resistance to freezing and thawing of the

cells (and mAbs) must be assessed. At various cloning and culture stages, the appropriate cell lines are frozen in liquid nitrogen.⁽¹⁵⁾

3.2. Phage Display Method:

Phage display, a cutting-edge or alternative technology created in 1985, is distinct from hybridoma technology.⁽⁹⁾ After Georg P. Smith's research on filamentous phage display, this display technique is currently frequently employed. It is also referred to as an alternate technique for producing small peptides and protein molecules.^(9,16) Additionally, the antibody gene in phage was generated and screened for the first time using lytic phage lambda.⁽¹⁶⁾



[Fig.13-Production of mAbs by Phage display platform]

The initial phase in this procedure is the separation of B-lymphocytes from the body's blood, which is followed by the separation of mRNA and its subsequent conversion to cDNA using PCR. This technique is coupled with the use of PCR to amplify the variable region segments and is followed by the cloning of the expressed immunoglobulin variable section of cDNA, producing the phage-displayed mAb variable region. This phage's IgG variable region can be converted into a library. It is also possible to construct a library of different human IgG variable region segments.^(9,17)

Isolation of B- lymphocytes:

The initial step is to isolate B-lymphocytes from human blood.⁽¹⁷⁾

Conversion into cDNA:

The next step is separating the mRNA from the B cells, which is followed by the conversion of the mRNA into Cdna.⁽¹⁷⁾

Amplification of specific variable region:

Both the heavy and light chains variable areas are amplified using PCR and the expressed portions of the variable region are then cloned as the next step.^(9, 17)

Cloning into vector:

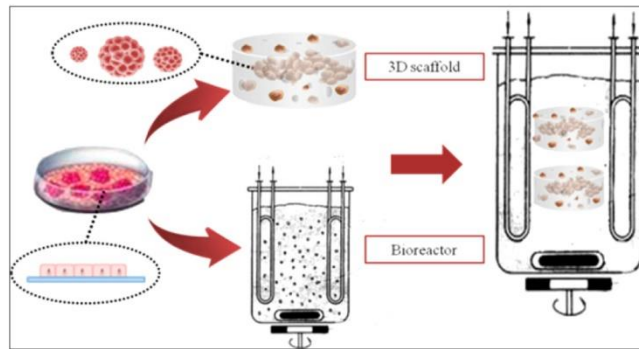
In order to infect E. coli, the bacteriophage (P111 protein) and phage protein are both used.⁽¹⁷⁾ Although this protein is present on the phage's surface, it has been discovered that the M13 phage contains the final molecule created by the fusion of antibody fragments and the P111 protein.⁽¹⁶⁾ When E. coli is used as a vector, various heavy- and light-chain bacteriophage regions begin to be produced. Then, a bacteriophage carrying E. coli is reinoculated using the variable sections of a given antigen.⁽⁹⁾

Library of phage displayed antibody:

The library of the particular phage that exhibits antibodies is created as a result of the isolation of cells bearing plasmids and sequencing.^(9,17) There are two distinct genetic systems available in this procedure for the expression of the antibody, including the direct insertion of the antibody genes into phage that is coupled to M13 phage protein III. The P111 protein and the antibody genes are delivered on the phagemid system, which is regarded as a distinct plasmid that contains the signal to assemble the phage particles for packaging the vector.⁽¹⁶⁾

3.3. IN VITRO METHOD:

Since immunisations are not necessary for the phage display method, which also uses the hybridoma technology, in vitro method is used to continue the process. According to certain sources, in vitro procedures refer to mammalian cell culture fermentation techniques carried out in bioreactors and continuous perfusion culture systems. This procedure is known as the tissue culture technology for cultivating hybridoma cells to release mAb. By growing the hybridoma cells in vitro using a variety of techniques, they are cloned for growth in order to achieve greater concentrations of mAb.



[Fig.14-Production of mAbs by In vitro method]

In addition to this, the in vitro method is popular because there are no ethical concerns because it does not involve immunisation procedures, and it is also preferred because the devices needed for this method are readily available on the market depending on the procedure used and the complexity, cost, and quantity of antibody obtained. Different types of containers are employed for both the cultivation of hybridoma cells and the storage of the generated antibodies. These containers include membrane-based and matrix-based culture systems, static and agitated cell suspension culture systems, and high density bioreactors, including hollow fibre bioreactors. Between 2000 and 2003, GSK Bio evaluated the in vitro mAb production process, relying heavily on hollow fibre bioreactors and suspension culture systems. This approach is thought to be reasonable and useful.⁽¹⁸⁾

Types of IN VITRO mAb production methods:

Cell Suspension culture system:

Static and agitated cell suspension culture is another name for this kind of culture system, which uses plastic cell culture equipment with T-flasks to keep the cells on hand in a stationary state as well as roller culture and spinner culture to allow for medium agitation. There are two types of cell suspension culture systems: stationary suspension culture systems and rotation suspension culture systems. This technique has the benefit of being applicable to both small research groups that require a narrow spectrum of mAb and professionals who require a broad spectrum of mAb as well as biopharmaceutical applications.

Membrane and matrix based tissue culture system:

Comparing this culture system to a cell suspension culture system, it produces a significant volume of mAb. In this system, there are distinct compartments with separate compartments for nutrients and cells. Semipermeable membranes are available for the transmission of nutrients since the membrane and matrix-based tissue culture system is in a separate compartment. Additionally, there is a particular gassing membrane called a gas permeable membrane that is used in membrane-based tissue culture systems for the diffusion of carbon dioxide and the delivery of oxygen. In a matrix-based tissue culture method, the complete nutrient medium and three-fourths of the production media are changed with new medium twice a week for the efficient synthesis of mAb. Depending on the process, the replacement of the production medium may differ. The benefit of this method is that higher amounts of mAb are secreted yet in a smaller volume as a result. The possibility of contamination in the mAb produced as a result of interaction with dead cells is another drawback to this approach. CELL line and micro PERM are two commonly utilised membrane-based technologies today.

Hollow fiber bioreactor:

This technique falls within the high density bioreactor category and was created to encourage the high cell density culture that secretes a bigger volume of mAb. The small, tube-like filters found in hollow fibre bioreactors are described as having preset cutoffs, resembling semipermeable membranes found in membrane-based tissue culture systems, and being organised and packed in cylindrical modules. Aeration for cell cultures is provided by hollow fibre cartridges, which are created by packing hollow fibres together. These fibres give a vast surface area for culture. The cells are only grown or cultured in the extra capillary space in this system, which has both intra capillary and extra capillary space. Additionally, intra capillary space is crucial because it allows nutrients and critical metabolites to freely perfuse between extra capillaries. In this culture technique, cells are grown in a porous three-dimensional matrix on each individual component, which is continuously refilled with new medium. As a result, the supernatant contains the secreted antibody, which can either be used directly without purification or purified and utilised.

There are two ways to carry out the in vitro procedure, notably

1. **Single harvest production, and**
2. **Multiple harvest production method**

- The generation of mAb in the single harvest production method depends on the stability of the grown cell line and is also close to or above the amount of production. In this instance, only the maximum allowed number is generated.
- Contrarily, continuous cultivation is used in multiple harvest production methods, and this should be specified as well as dependent on the product's consistency and stability. It also depends on a number of variables, including the type of monitoring, the frequency necessary, the expression system, the nature of the antibodies, and the duration of the continuous cultivation phase.⁽¹⁸⁾

3.4. IN VIVO METHOD:

Although the in vitro method is frequently utilised, there are some circumstances when it is discovered to be useless because the characteristics and properties of hybridoma cells differ as a result of the diversity of mAb formation. Other alternative ways are taken into consideration after utilising in vivo methods; however for a legitimate reason only in vivo methods are suitable.



[Fig.15-Production of mAb by In vivo method]

✚ For reasons like the cells' inability to adapt to in vitro conditions, the cell line's inability to maintain mAb production, the conditions where purification leads to decreased antibody level, as well as the loss of utility of cultured cells due to contamination, there is a need for scientific justification because it is thought to be a more painful method to the animals. In the in vivo approach, which is somewhat similar to the in vitro method, the hybridoma cells are created and then injected into the peritoneal cavity of mice for cell proliferation, after which fluid is collected in the mice's abdomen. Mice experience pain as a result of the fluid buildup caused by the hybridoma cells' increased rate of cell division. Since the fluid is referred to as ascites fluid, this method of producing mAb might also be called the ascites method. Because this procedure causes distress to mice and because mice are more necessary in this method as more antibody is needed, the usage of mice is generally limited.⁽¹⁸⁾

The steps involved in IN VIVO production method :⁽¹⁸⁾

3.4.1. Priming:

✚ The mice's peritoneal cavity is primed before the hybridoma cells are injected there. Priming drugs, such as Pristane or Freund's agent, are injected intraperitoneally (IP) during this process. There is a specific volume that must be used, for instance, Pristane should not be administered in amounts greater than 0.3 ml because doing so causes distress due to its irritating nature. Because it is more unpleasant, Freund's agent is not generally advised.

3.4.2. Inoculation of hybridoma cells:

✚ In this step, hybridoma testing is carried out, in which hybridoma cells are examined for the production of particular antibodies before being injected into the mice's peritoneal cavity. The number of hybridoma cells to be inoculated should be between 10^5 and 10^7 , but no more than 0.5ml should be used.

3.4.3. Production of ascites fluid:

✚ When hybridoma cells are injected, a tumour develops and ascetic fluid gathers within it. The formation of this ascetic fluid and a huge cell inoculum causes the mice's short lifespans or low survival rates. Since the creation of ascetic fluid is extremely painful, this is the step that causes the mice to feel distressed and uncomfortable, hence the mice should be checked on by the researcher frequently, at least twice a day. There are some symptoms of distress during this stage in the mice, including abnormal breathing like rapid or even shallow breathing, hyperthermia, hypothermia, rapid weight loss, as well as diarrhoea and constipation, so if there are any serious symptoms, medical assistance is advised. The mice experience difficulty getting to food or water because of the increased abdominal distension than during pregnancy.

3.4.4. Collection of ascites fluid:

✚ The survival rate of mice declines as ascetic fluid production rises, it is important to harvest ascites fluid as soon as feasible in order to lower mouse mortality. The weight cannot exceed 20% of the normal weight for the patient's age and sex. Therefore, the collection procedure should be completed before abdominal discomfort arises. Before tapping, the mouse is given anaesthesia by a skilled individual because it is a painful procedure. Then, the mice are held while a 20- to 21-gauge needle is used to tap its abdomen, collecting the ascetic fluid in a collection tube. Two abdominal taps should be performed, while three can be done in cases of euthanasia. Regular observation is also required following each tap, and veterinary staff is advised if there is any obvious acute suffering.

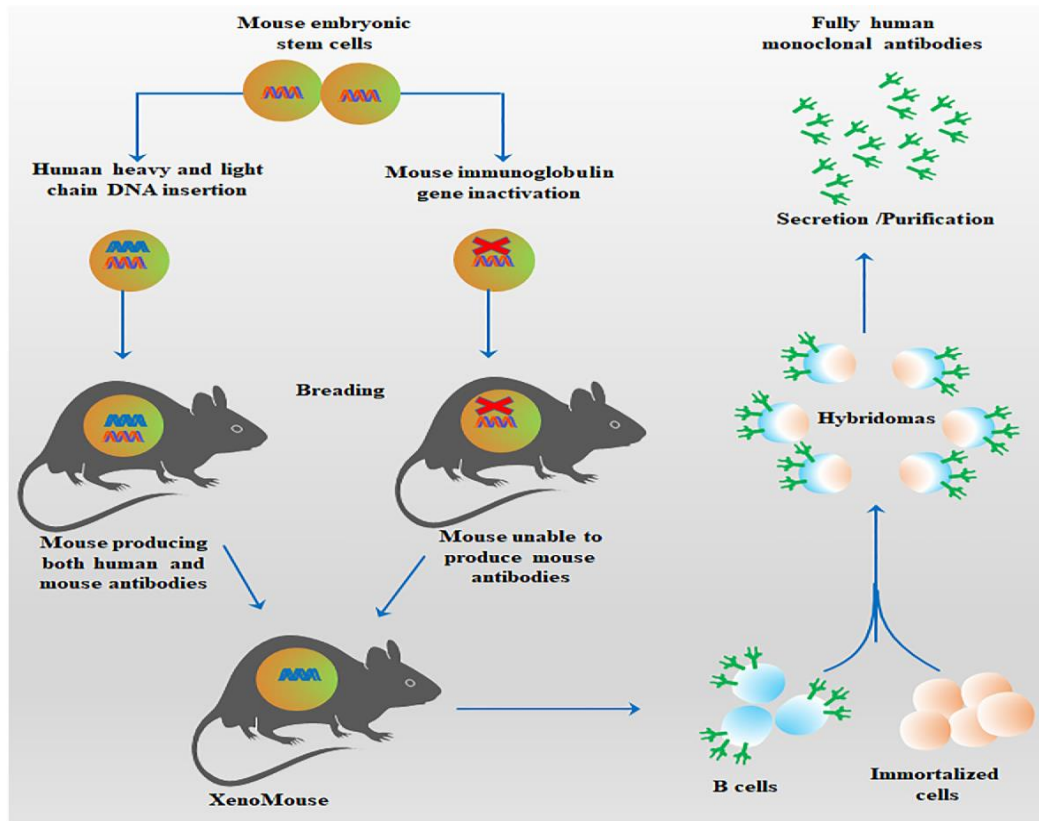
3.4.5. Purification of Mab:

✚ The ascetic fluid contains the secreted antibodies, but in order to obtain it, a purification process is carried out because it might also contain pollutants and other chemicals such as cytokines released by hybridoma cells. Centrifugation and filtration are used in the purification procedure to remove bigger cell debris and entire cells, followed by ion exchange chromatography to remove any nucleic acids, and affinity chromatography to acquire the desired antibodies. Alternative techniques for obtaining antibodies include precipitation, in which the mAb is precipitated, and electrophoresis.

3.5. Transgenic mice method:

The most cutting-edge technique for producing human mAb from trans-chromosome mice is the transgenic mice approach because the immunoglobulin of transgenic mice has human immunoglobulin expressing genes in both the heavy and light chain.

In order to create human mAbs using traditional cell culture techniques, mice that have been genetically engineered to produce human antibody repertoires were first described. Transchromosomal engineering is used to modify the endogenous mouse immunoglobulin heavy chain and immunoglobulin light chain loci. Human immunoglobulin heavy and light chains were encoded by transgenes. Over the past few decades, progress has been made in getting transgenic mice to express various V gene segments, with a growth in the number of potential repertoires of the retrieved mAbs. Although immune responses are occasionally less robust in transgenic mice compared to those seen in normal mouse strains used to generate mouse mAbs, a generation of transgenic animals producing human mAbs with varied heavy chain isotopes was obtained.



[Fig.16-Production of mAb by transgenic mouse method]

Despite the aforementioned restrictions, about 50 human mAbs are now being tested in clinical studies, and about six different transgenic human mAbs have been approved for marketing. This method of human immunoglobulin expression in transgenic mice minimises unfavourable human anti-mouse antibody reactions while maintaining the advantages of standard murine cell culture for the production of potential therapeutic medicines.^(19,20)

3.6. Production of mAb from plants:

A recent finding indicates that plants are now used to manufacture mAbs. Therefore, plants can be utilised to produce mAbs on a massive scale, and since transgenic plants function as bioreactors, they can also be used to cultivate mAbs in vitro. Most often, it is grown using in vitro methods. While the method of extracting mAb from plants is quite safe, it has a medium production time and unknown therapeutic risk compared to other methods of mAb derivation. It also has low production and maintenance costs and a high yield. This approach also has certain drawbacks, including an unpredictable culture parameter and a high risk of contamination from bacteria or pollen. The process of purification, which can be carried out using protein A or protein G-based affinity chromatography, is crucial.^(21,22)



Production of Anticancer Monoclonal Antibodies

- Major problems:**
- Low level of expression
 - High level of degradation

Large-scale production:

- Cost
- Turnaround time
- Regulatory requirements

Cancer

[Fig.16- Production of mAbs from plant]

3.7. ADVANTAGES OF mAb

- + Production can take place both in vitro and in vivo.
- + One antigenic determinant.
- + High repeatability.
- + It is possible to create high avidity.
- + It is feasible to produce cell lines specific to each ingredient in a mixture.
- + Antiserum titre was set to a very high value.
- + One can identify cross-reaction.
- + It is feasible to radiolabel and fluorescently conjugate.
- + It is not necessary to vaccinate or maintain bleeding in farm or animal animals.⁽²³⁾
- + Pure one species of molecules exclusively.⁽²⁴⁾

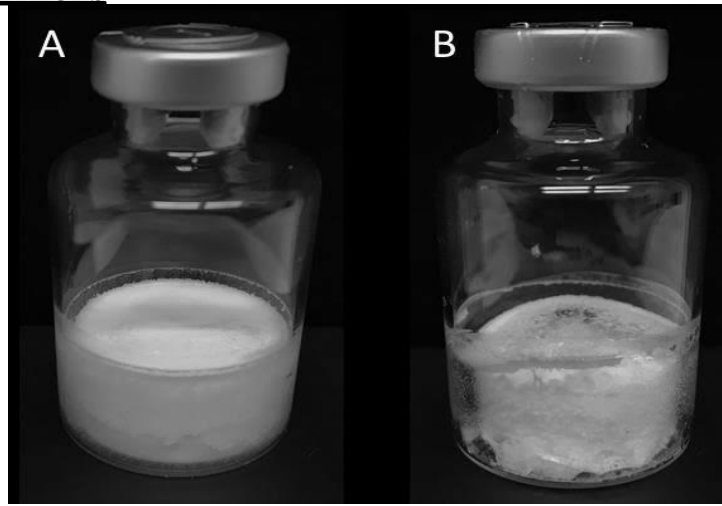
3.8. DISADVANTAGES OF mAb

- + The project took between 6 and 9 months to complete, was expensive, and required a lot of work to construct.
 - + It's possible that small peptide and fragment antigens are not the best antigens. The initial antigen might not be recognised by a monoclonal antibody.
 - + Contamination may affect hybridoma culture.
 - + The System is not well established for other animals and is only well developed for a few mammals.
 - + During the fusion process, more than 99% of the cells do not survive, which limits the number of effective antibodies that may be made against an antigen.
- It has the potential to produce immunogenicity.⁽²⁵⁾

3.9. LIMITATIONS OF mAb

- + -Mouse myeloma cells cannot be replaced by compatible human myeloma cells.
- + -The fusion of mouse myeloma cells and human lymphocytes is extremely fragile.
- + -The process takes longer and requires more money up front.
- + -In the case of mAbs, as well as in the case of traditional antiserum, the energy of binding to an antigen is slow.
- + -It differs from the typical double immunodiffusion technique.
- + -Humans cannot be immunised against antigens for ethical reasons.⁽²³⁾

3.10. EVALUATION METHODS



(Fig.17-mAbs in the form of (A) freeze dried solid (B) Liquid form)

❖ **Characters:**

Clear or barely opalescent, colourless or barely coloured liquids are defined as liquid preparations. Products that have been freeze-dried are solid friable masses or white or faintly tinted granules. They exhibit the same properties as liquid preparations after reconstitution.

❖ **Identification:**

When necessary, the identification is established by comparing the product with the reference preparation using relevant validated methods. The assay helps with identification as well.

❖ **Appearance:**

In terms of degree of opalescence and degree of colouring, liquid or reconstituted freeze-dried preparations adhere to the limitations permitted for the specific product. Except if approved and justified, they are free of visible particles.

❖ **Solubility:**

Freeze-dried preparations entirely dissolve in the recommended volume of reconstituting liquid within the authorised timeframe for the specific product.

❖ **PH:**

It complies with the restrictions set forth for the specific product.

❖ **Osmolality:**

Unless otherwise justified and authorised, at least 240 momol! Kg.

❖ **Total protein:**

It complies with the restrictions set forth for the specific product. 90 to 110 percent of the amount specified on the label, but not more than 100 g/L unless otherwise justified and approved.

❖ **Molecular-size distribution:**

A suitable approach, such as size-exclusion chromatography, is used to determine the molecular-size distribution. It complies with the restrictions set forth for the specific product.

❖ **Molecular identity and structural integrity:**

Various tests can be employed to show molecular identity and structural integrity of the monoclonal antibody, depending on the kind of antibody, its microheterogeneity, and its isoforms. These tests could involve oligosaccharide mapping, monosaccharide content, isoelectric focusing, ion-exchange chromatography, hydrophobic interaction chromatography, peptide mapping, and mass spectrometry.

❖ **Purity:**

Testing for contaminants relevant to processes and products is done using appropriate, approved methodologies. They may be omitted from the final lot as long as testing for process-related contaminants on the active ingredient or on the final bulk have been completed with positive results.

❖ **Stabiliser:**

It complies, when necessary, with the restrictions set for the specific product. By using an appropriate physico-chemical approach, determine the stabiliser's quantity. The preparation includes between 80 and 120 percent of the quantity listed on the label, but not less.

❖ **Water:**

Products that have been freeze-dried adhere to the standards established for that specific product. 3 percent at most.

❖ **Sterility:**

The product should pass the sterility inspection.

❖ **Bacterial endotoxins:**

It complies with the restrictions set forth for the specific product.

❖ **Tests applied to modified antibodies:**

Depending on the type of modification, the appropriate tests are run.

❖ **Pyrogens:**

It passes the pyrogens test unless otherwise justified and authorised. Inject 1 mL per kilogramme of the rabbit's body mass unless otherwise directed.

❖ **Assay:**

Conduct an appropriate biological assay in comparison to the reference preparation. The assay is created, and the findings are calculated, using standard methods. ⁽²⁶⁾

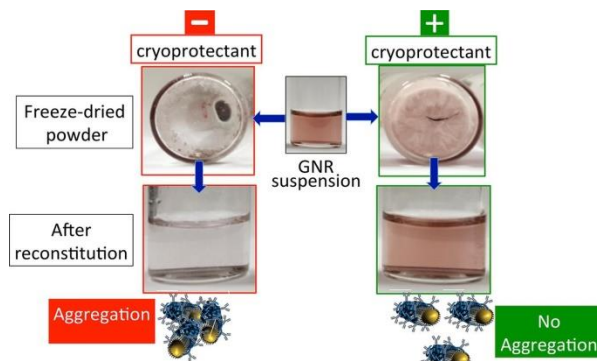
3.11. STORAGE



[Fig.18-Storage]

STORAGE CONDITIONS FOR mAb

Kept at the specified temperature range and protected from light. It is best not to let liquid preparations freeze. Date of sterile filtration, date of filling (for liquid preparations), or (if applicable) date of freeze-drying are used to calculate the expiration date. ⁽²⁶⁾



(Fig.19- Frozen mAb product)

3.12. LABELLING

- ✚ Where appropriate, the number of units per millilitre.
- ✚ The volume of protein in each container.
- ✚ The volume of monoclonal antibody in each container.
- ✚ The amount of preparation in the container for liquid preparations.

For freeze-dried preparations:

- The name and quantity of the reconstitution liquid to be added.
- The time frame for using the monoclonal antibody after reconstitution.
- ✚ If necessary, the dilution to be produced prior to product usage. ⁽²⁶⁾

3.13. APPLICATION OF mAb

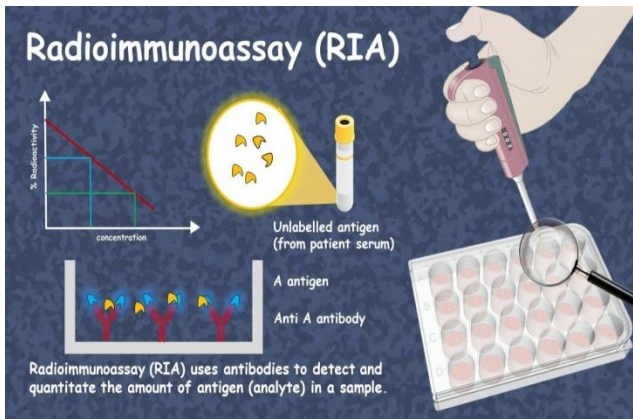
There are several applications for monoclonal antibodies, including disease prevention, detection, and treatment. ⁽²⁷⁾

3.13.1. DIAGNOSTIC APPLICATION : ⁽²⁸⁾

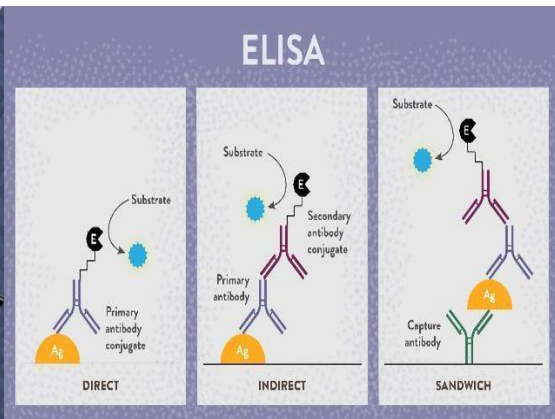
The laboratory diagnosis of many diseases has been revolutionised by monoclonal antibodies. mAbs may be used for this purpose as diagnostic reagents for biochemical analysis or as instruments for diagnosing diseases through imaging.

mAbs in biochemical analysis:

In the laboratory, radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) routinely use diagnostic testers based on the usage of MABs as reagents. Many commercially available diagnostic kits that use MABs have emerged in recent years. It is now helpful for disease diagnosis across the board.



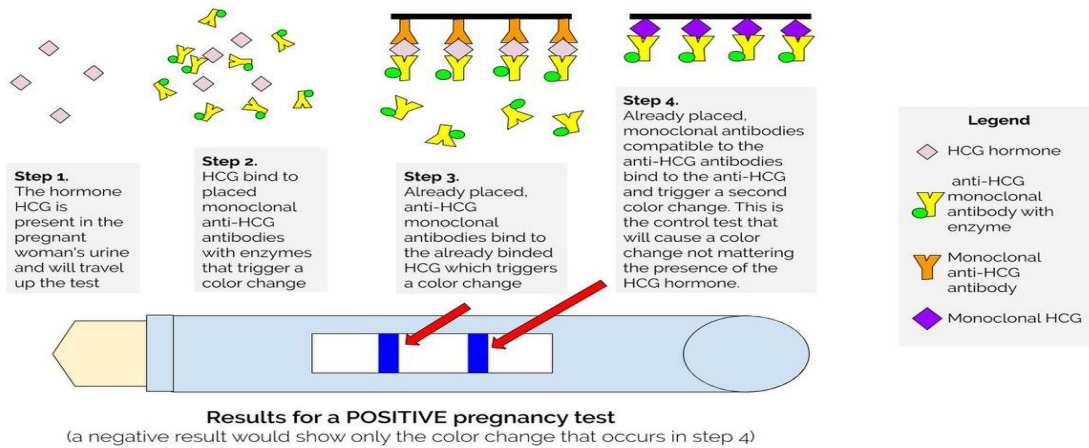
[Fig.20-RIA]



[Fig.21-ELISA]

3.13.1. A. Pregnancy:

Human chorionic gonadotropin levels in the urine can be used to determine pregnancy.



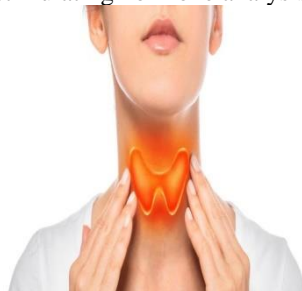
[Fig.22-Pregnancy test]

3.13.1. B. Cancer:

Plasma carcinoembryonic antigen is a marker for colorectal cancer, whereas prostate specific antigen is a marker for prostate cancer. Estimating tumour markers is helpful for cancer prognosis in addition to diagnosis. Following treatment, a progressive decline in a particular tumour marker is seen along with a decrease in tumour size.

3.13.1. C. Hormonal disorders:

Thyroxine, triiodothyronine, and thyroid-stimulating hormone analysis for thyroid diseases



[Fig.23-Thyroid disease]

3.13.1. D. In cardiovascular disease:

With the aid of radio-labelled antimyosin mAb, the site and extent of heart injury can be detected. The antimyosin monoclonal antibody (mAb) binds to intracellular myosin that has been made visible as a result of cardiac necrosis and is specific for human myosin. To localise the atherosclerotic lesions using imaging techniques, atherosclerosis mAb radiolabelled against platelets can be utilised.



[Fig.24-Cardiovascular disease]

3.13.1. E. In malaria:

The current method of diagnosing malaria infection involves microscopic inspection of blood smears, which is efficient but labour- and time-intensive. Although rapid and automatable immunological methods for plasmodium detection, like ELISA, do not discriminate between detecting antiplasmodium antibodies in the blood of infected people.



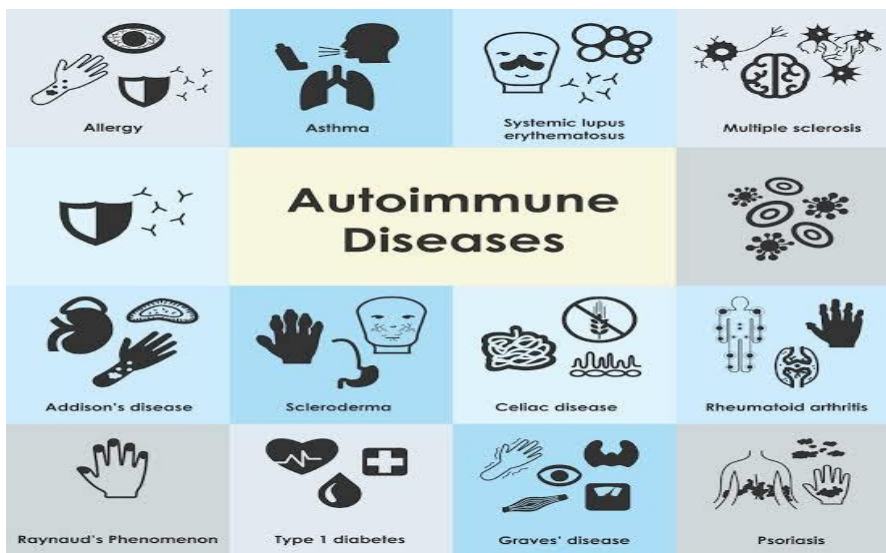
[Fig.25-Malaria]

The malaria-causing organism Plasmodium falciparum is identified via a diagnostic process that employs a DNA probe as a means of detection.

3.13.2. THERAPEUTIC APPLICATIONS:

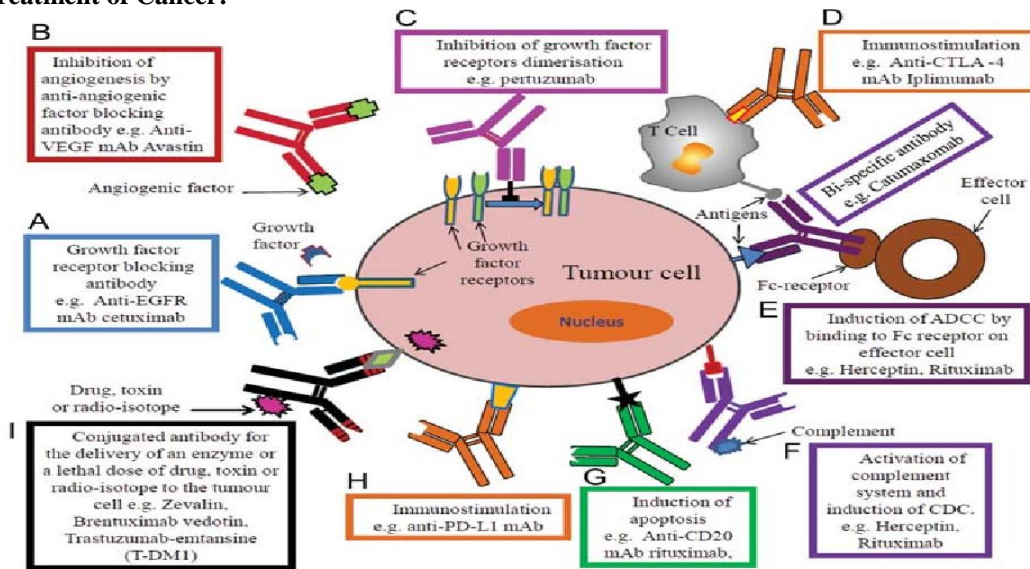
3.13.2. A. Treatment of autoimmune diseases:

mAbs may be able to reduce immune system activation in autoimmune disorders or following organ donation. Numerous inflammatory diseases, including psoriasis, rheumatoid arthritis (RA), Asthma, Graves’ disease, celiac disease, Type 1 diabetes, Systemic lupus erythematosus, Allergy, juvenile arthritis, Crohn’s disease, and multiple sclerosis, Addison’s disease, Raynaud’s phenomenon have demonstrated promising therapeutic applications of mAbs.⁽²⁹⁾



[Fig.26-Autoimmune disease]

3.13.2. B. Treatment of Cancer:



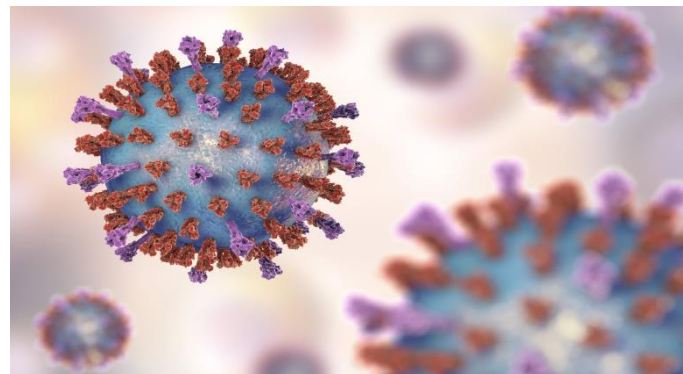
[Fig.27-Cytotoxicity Mechanism]

- Through antibody-dependent cell cytotoxicity, monoclonal antibody-mediated treatment draws in cytotoxic cells (monocytes and macrophages).⁽³⁰⁾
- mAbs bind complement proteins, which results in direct cell cytotoxicity that is complement-dependent in nature when used to treat cancer.⁽³¹⁾
- In order to successfully stop the growth of tumour cells, certain monoclonal antibodies have been shown to block growth factors by attaching to and inhibiting growth factor receptors.⁽³²⁾

3.13.2. C. Treatment of infections:



[Fig.28-Hepatitis virus]

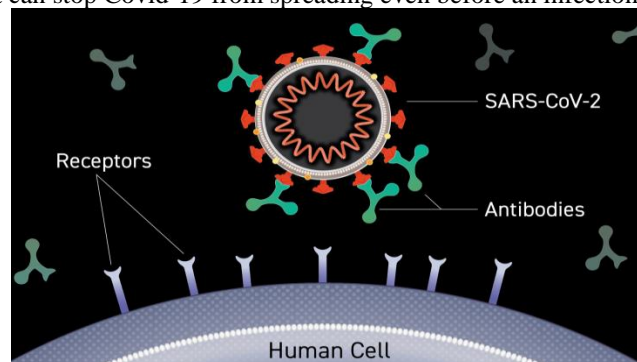


[Fig.29-RSV]

- Used to treat Infection with the respiratory syncytial virus in children and hepatitis.

3.13.2. D. Treatment of SARS-CoV-2 coronavirus:

- The creation of cures that can stop Covid-19 from spreading even before an infection occurs.⁽³³⁾



[Fig.30-mAb against COVID]

3.13.3. INVESTIGATIONAL AND ANALYTICAL APPLICATION:

3.13.3. A. Protein purification:

✚ Every protein can create monoclonal antibodies. Furthermore, the purification of the protein against which it was grown is easily accomplished using the MAb that was so created.

✚ By joining mAbs to cyanogen bromide activated Sepharose (chromatographic matrix), mAbs columns can be created. The immunoaffinity approach of protein purification makes excellent use of the immobilised mAbs.

✚ Using mAbs for protein purification has a few benefits. These include a high level of purification, very effective elution from the chromatographic column, and the ability of the mAb to bind specifically to the required protein.⁽²⁵⁾



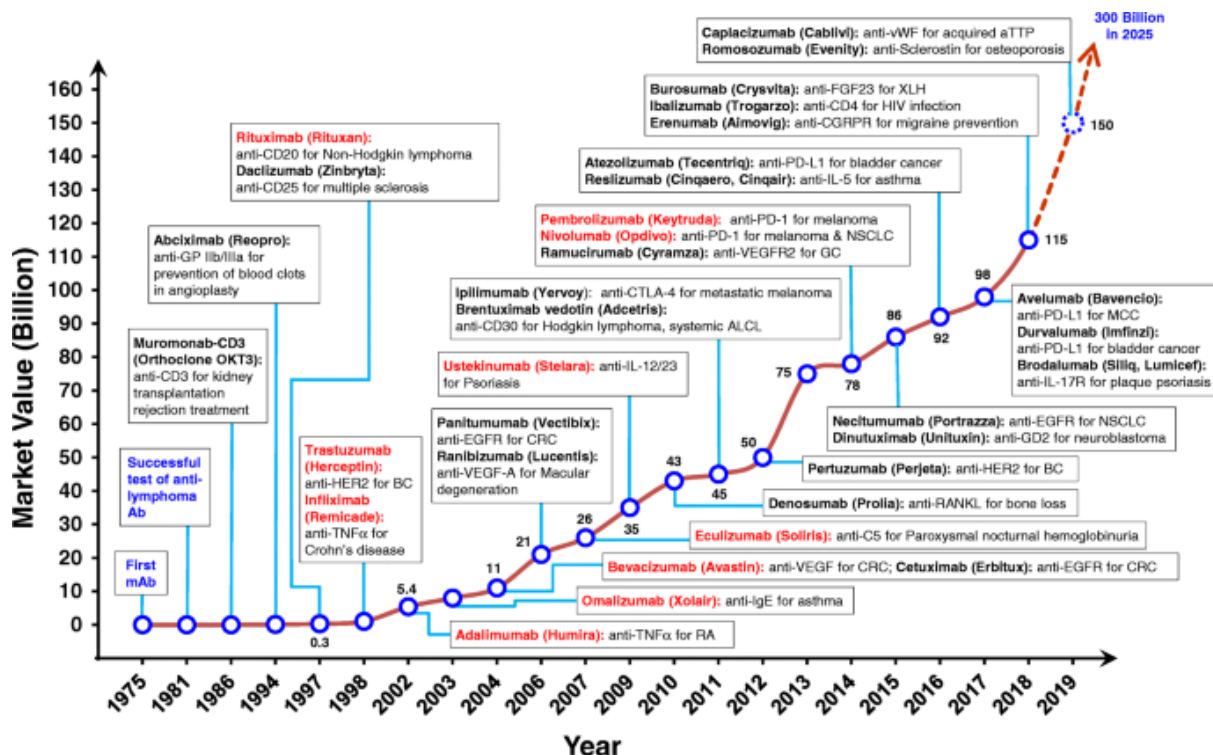
[Fig.31-Column protein purification]

3.13.3. B. Identification and isolation of lymphocytes phenotyping:

✚ mAbs specific for various lymphocytes subpopulation express characteristic pattern of membrane protein. This characteristic can be utilized for identification of various stages of lymphocytes differentiation, e.g. human and mice in both CD8 membranes expressed by T helper cells.

MARKET SIZE OF mAbs:

✚ A timeline from 1975 illustrating the effective creation of therapeutic antibodies and their uses. From 1981 to 1986, a lot of biotech businesses were established, many of which touted antibodies as "magic bullets" against cancer. The estimated market value of mAb therapies in each indicated year is represented by the height of the line and the numerical annotations (given in billions of US dollars). The top 10 antibody medications in 2018 are represented by antibodies in red. Breast cancer; aTTP, acquired thrombotic thrombocytopenic purpura; Ab, antibody; ALCL, systemic anaplastic large-cell lymphoma; Cluster of Differentiation, or CD Colorectal cancer, calcitonin gene-related peptide, and calcitonin gene-related peptide receptor Cytotoxic T-lymphocyte-associated protein 4 is known as CTLA-4. GD2, disialoganglioside GD2, HER2, human epidermal growth factor receptor 2; IgE, immunoglobulin E; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; GC, gastric cancer; mAb, or monoclonal antibody; IL, or interleukin; IL-17R, or interleukin-17 receptor; Merkel-cell carcinoma, non-small cell lung cancer, rheumatoid arthritis, PD-1 programmed cell death protein 1, PD-L1 programmed deathligand 1, TNF, tumour necrosis factor ; VEGF-A, vascular endothelial growth factor A; VEGFR2, vascular endothelial growth factor receptor 2; vWF, von Willebrand factor; XLH, X-linked hypophosphatemia. RANKL, receptor activator of nuclear factor kappa-B ligand.⁽³⁴⁾



[Fig.32-List of mAbs available in the global market]

The market for mAbs was valued at 237.61 billion in 2023, and by 2030, it is anticipated to reach 494.53 billion. North America is the biggest market for mAbs, whereas Asia, Pacific, is the market with the greatest growth.

5. Conclusion

Monoclonal antibodies are effective biological medicines for treating a variety of illnesses, including autoimmunity, cancer, and asthma. They have excellent specificity and affinity binding capabilities. Due to their excellent sensitivity and specificity, they are frequently utilised in immunodiagnostic assays.

In the field of cancer treatment, monoclonal antibodies (mAbs) are a highly targeted and efficient alternative therapy. They are created utilising hybridoma technique, which concentrates on one antigen's epitope rather than several. Applications for monoclonal antibodies in diagnostics include immunohistochemistry, ELISA, and western blotting. More advancement is required for a variety of diagnostic and therapeutic uses. Although monoclonal and polyclonal antibodies show promise for analytical applications, the moral implications of antibody manufacturing must be taken into account.

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