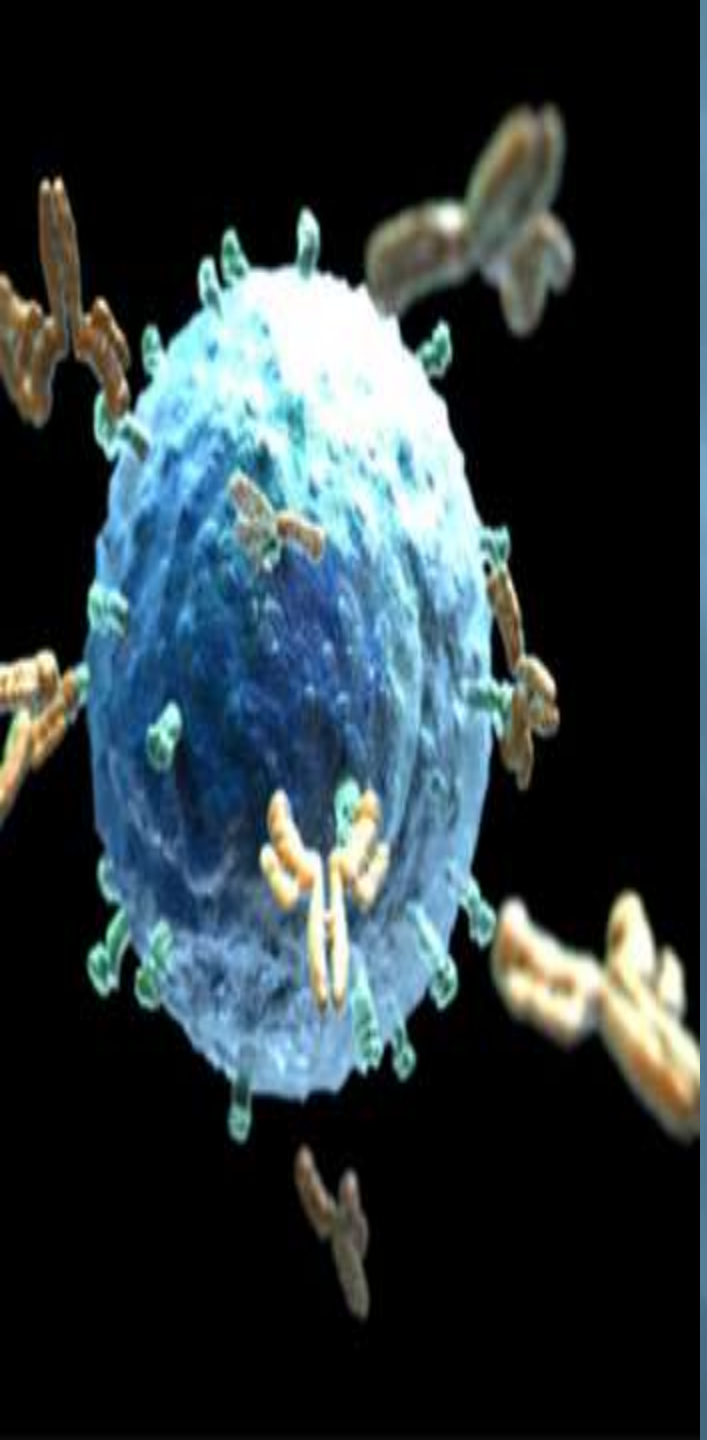

MONOCLONAL ANTIBODIES
….As Drug Carriers

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INTRODUCTION

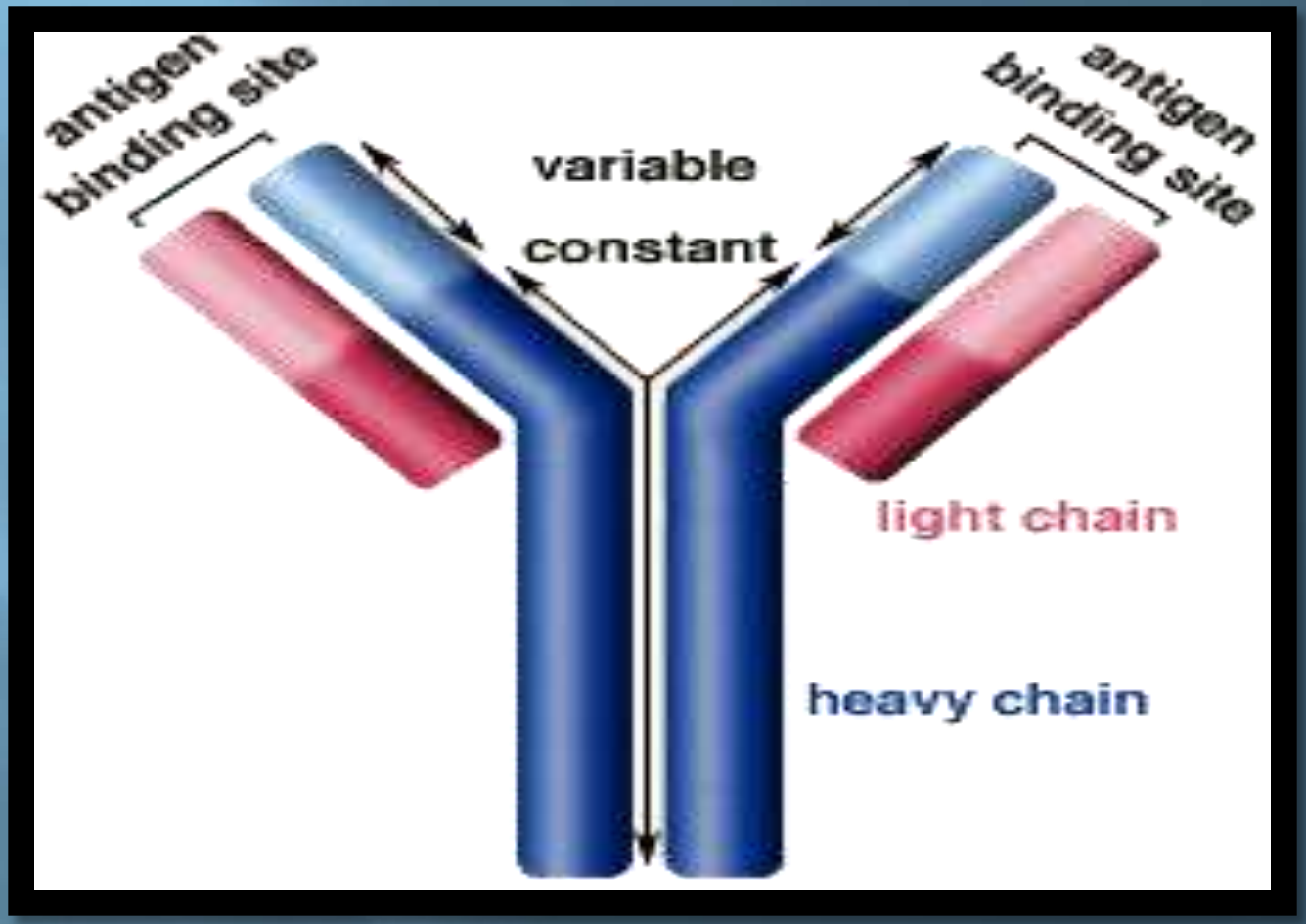
- **Antibodies** are serum glycoproteins synthesised and secreted by plasma cells which are derived from B-lymphocytes , stimulated in response to foreign substances called Antigens.

- These are also called as '*magic bullets*' since they accurately target and neutralize the antigens.

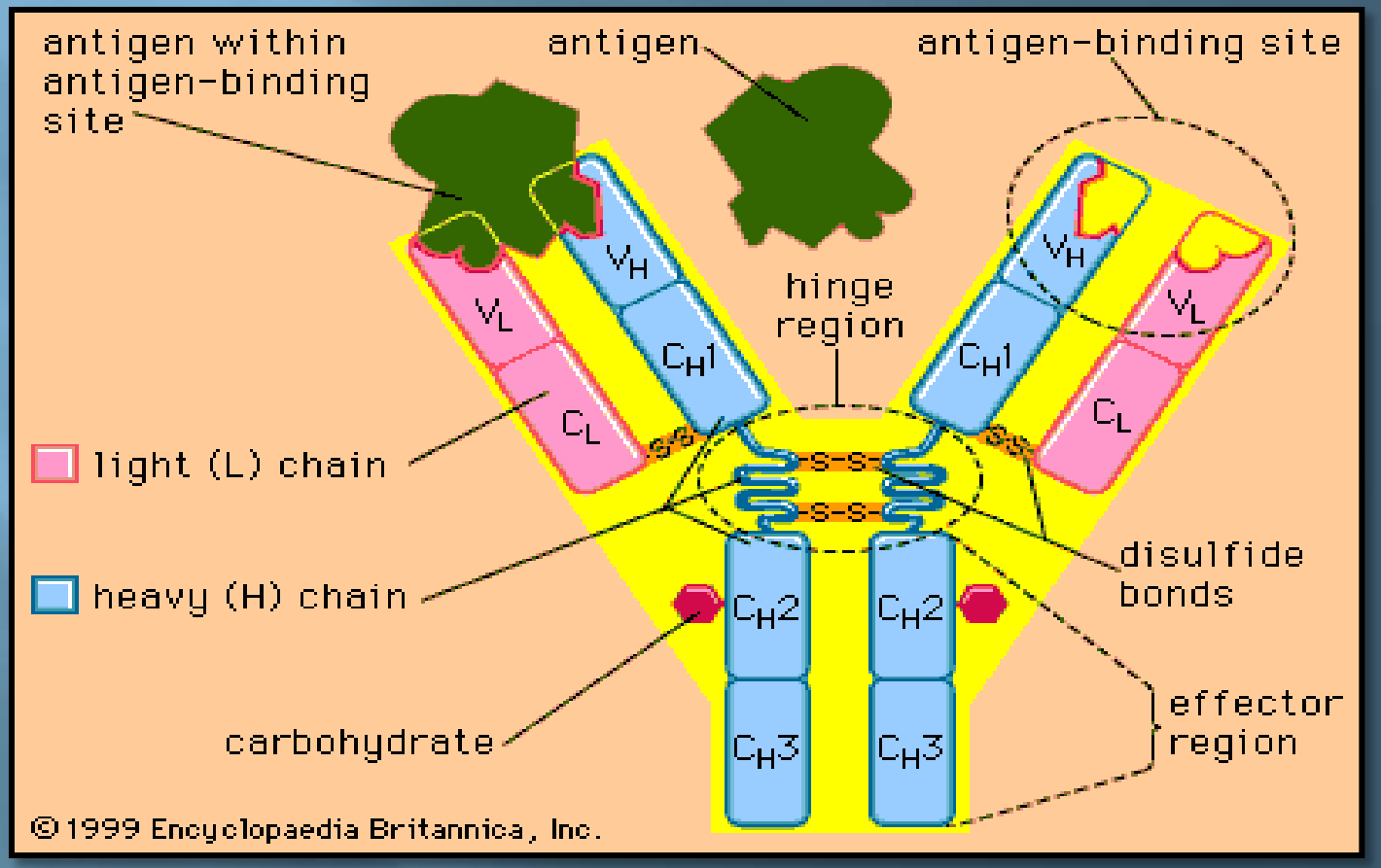
- Antibodies are extremely specific in their interaction and react with proper antigen only.

- These have vast applications in detection of infectious agent and serological reactions for diagnosis and curative purposes.

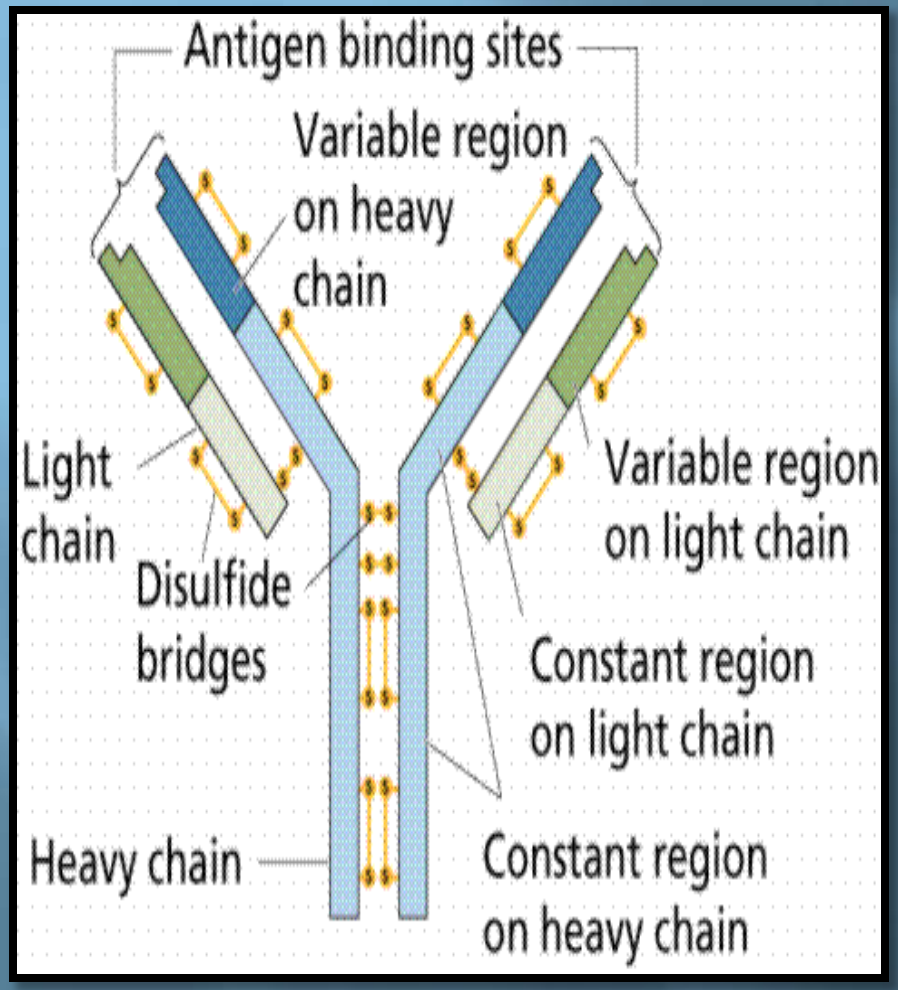
STRUCTURE OF ANTIBODY

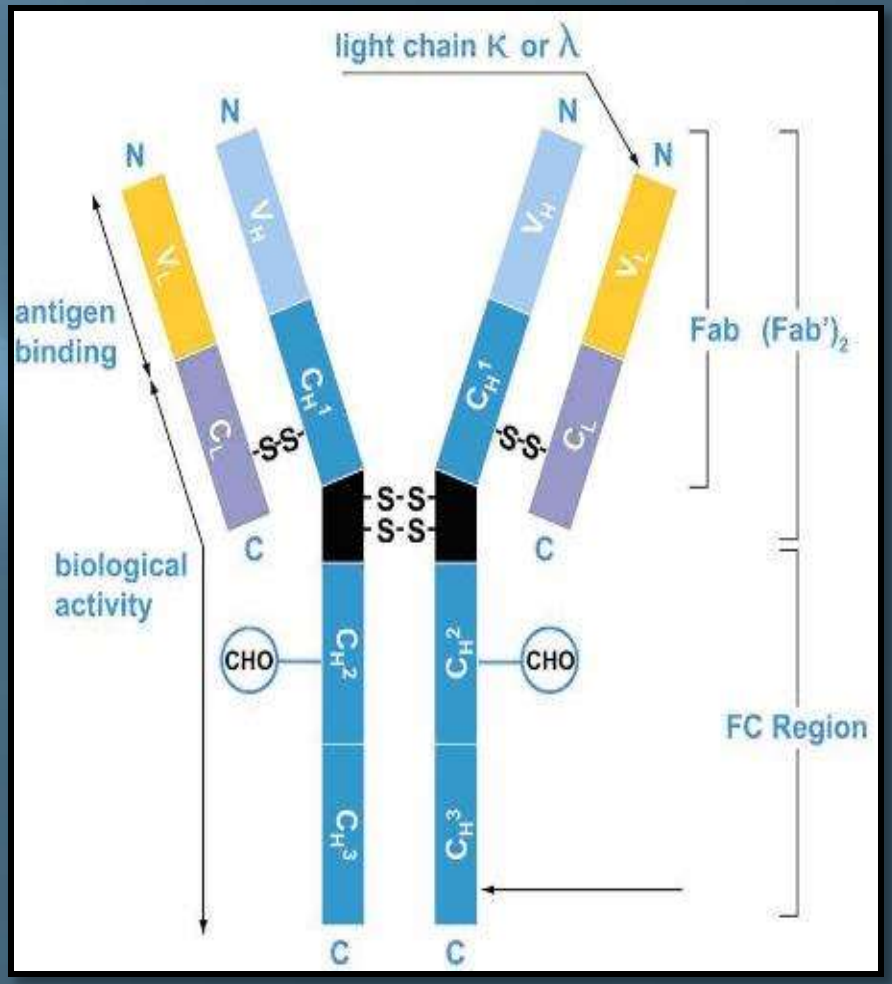


THE FOUR-CHAIN STRUCTURE OF AN ANTIBODY OR IMMUNOGLOBULIN MOLECULE



STRUCTURE OF ANTIBODY





- Normally immune serum will not only have specific antibodies, but also mixture of other antibodies.

- Thus, if antibodies are to be exploited for therapeutic and diagnostic purposes, their heterogenicity is to be overcome.

- Simply, this means that, to isolate *one* B cell and allow its cultivation to form a clone which will synthesize and secrete homogenous antibodies.

- But B cells are mortal, so they cannot be cultivated indefinitely.

- To overcome the problem of mortality, ***Caesar Milstein*** and ***Georges Koehler*** came up with the idea of fusing B cells with ***Myeloma cells*** to obtain a hybrid, called as *HYBRIDOMA* and the production of these is known as Hybridoma technology.
- For this work they were awarded the **Noble prize in the year 1984.**

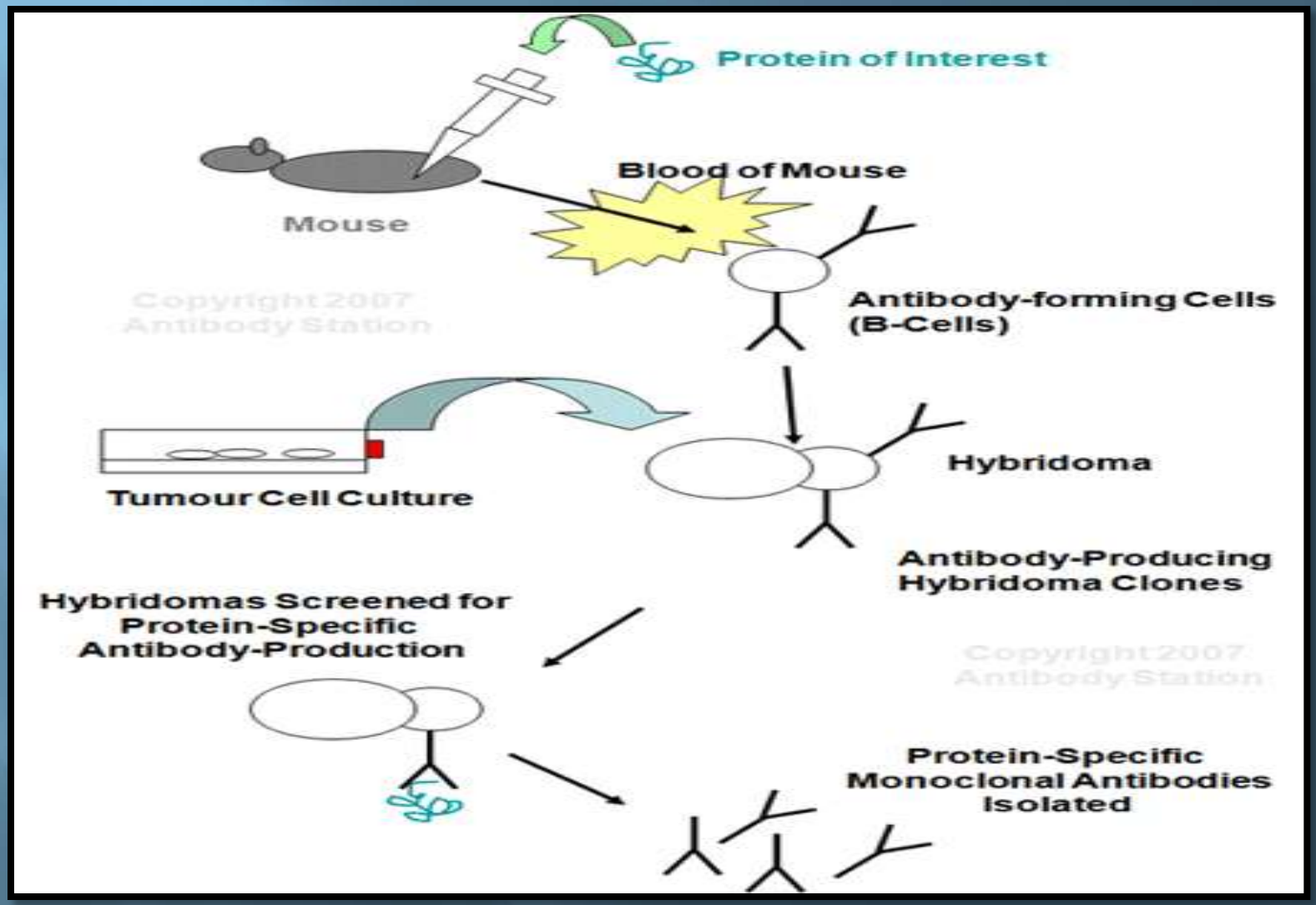
Monoclonal Antibodies:

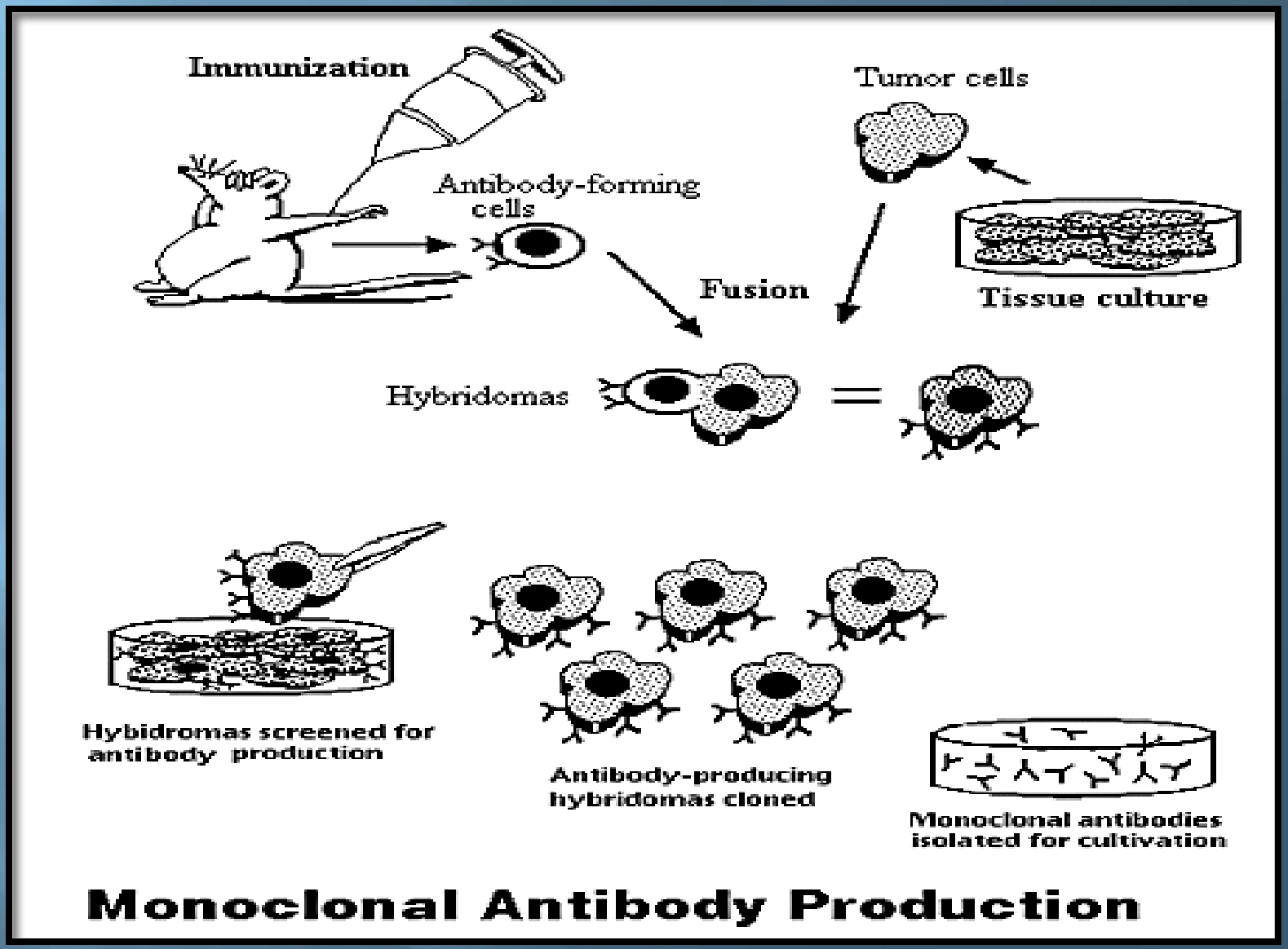
An antibody is called as monoclonal when each immunoglobulin is produced by a single clone of cells and hence is identical to every other molecule in the preparation.

- MCAbs therefore hybrids of ***antibody*** producing ***spleen cells*** and ***immortal myeloma cells.***

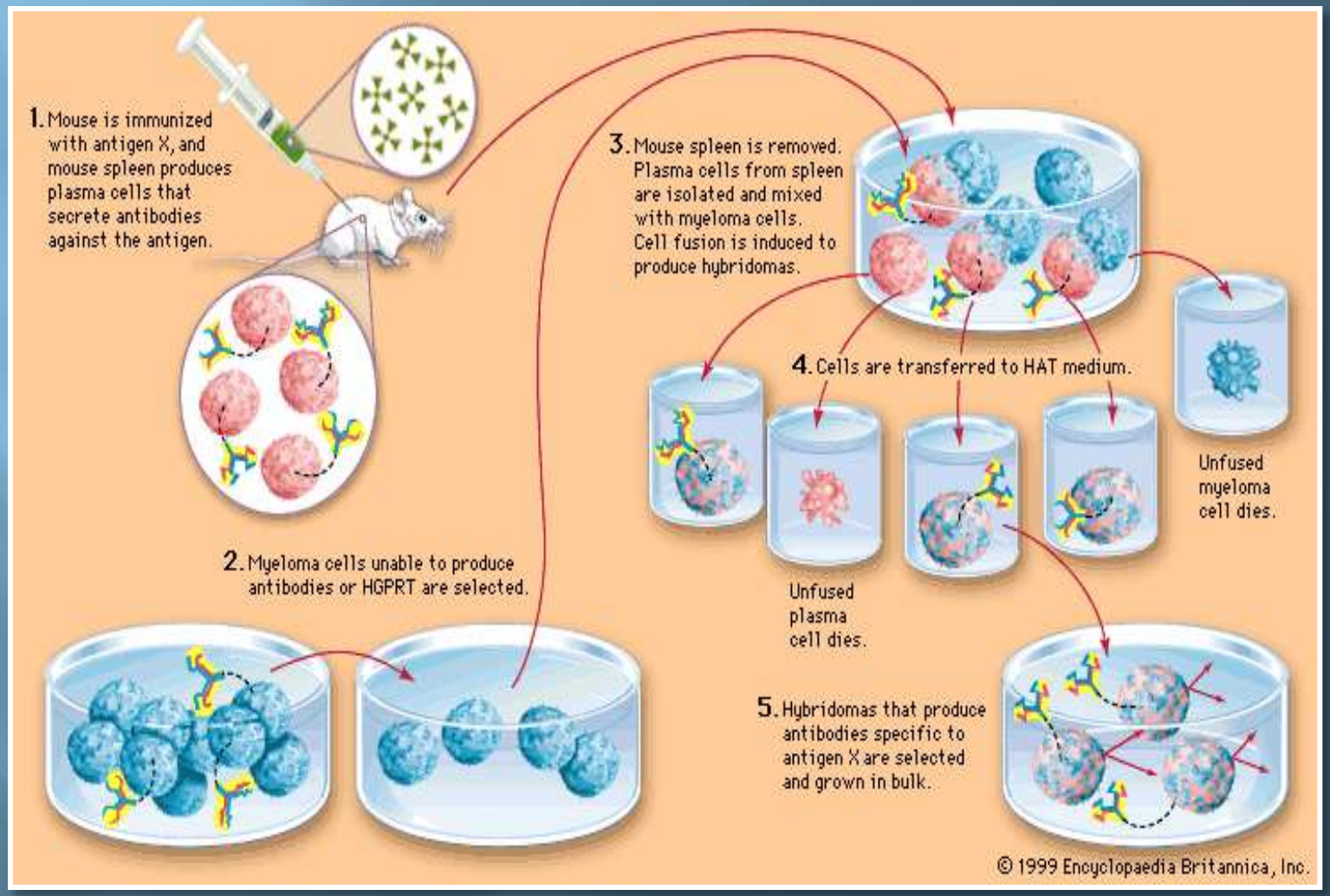
- The hybridoma is made by fusing a lymphocyte (B cell) with a myeloma cell.
- Presence of single antigenic determinant is the useful feature of the monoclonal antibodies.
- MCA bind with only one type of epitope on the antigens

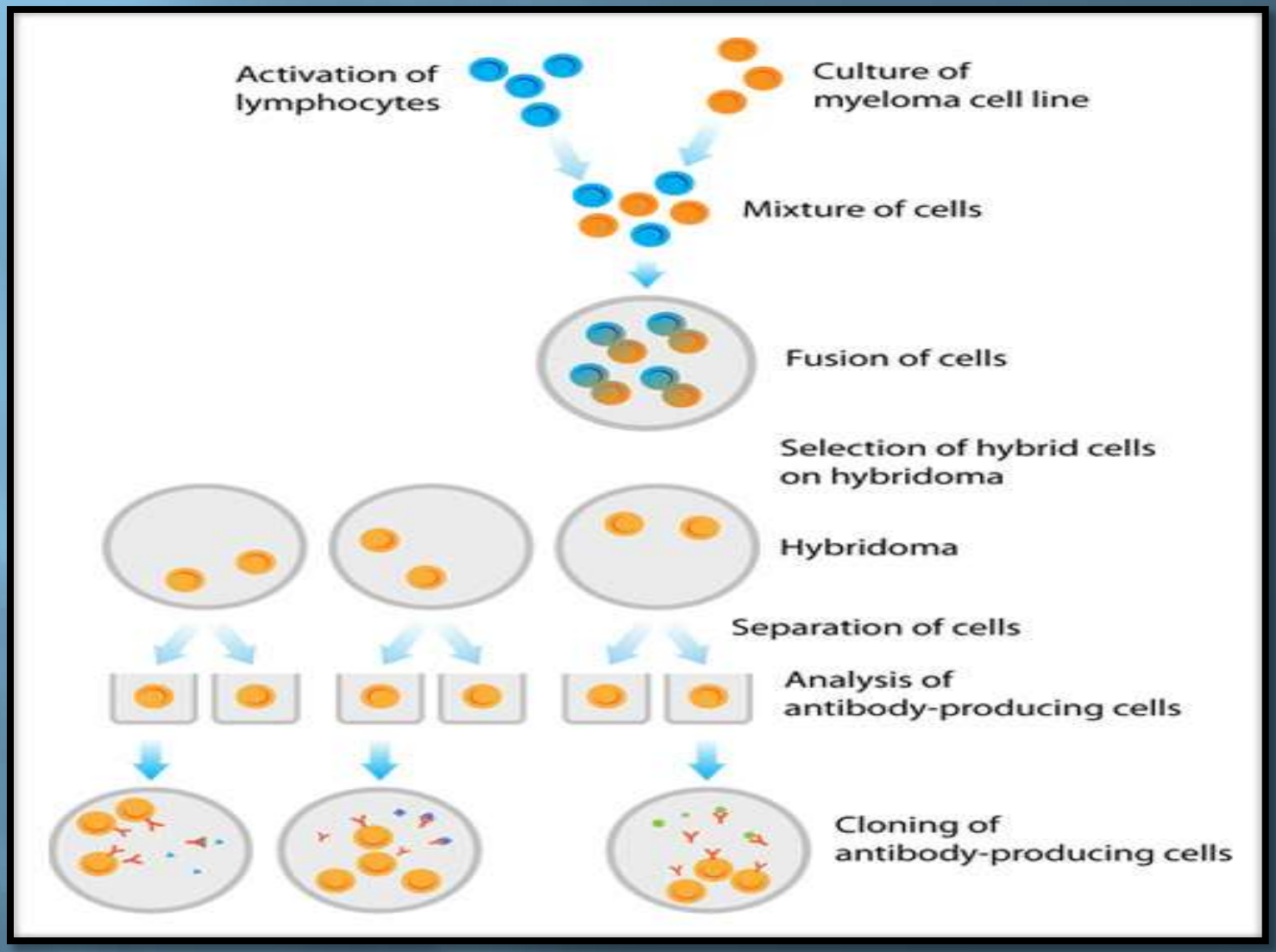
MONOCLONAL ANTIBODY PRODUCTION

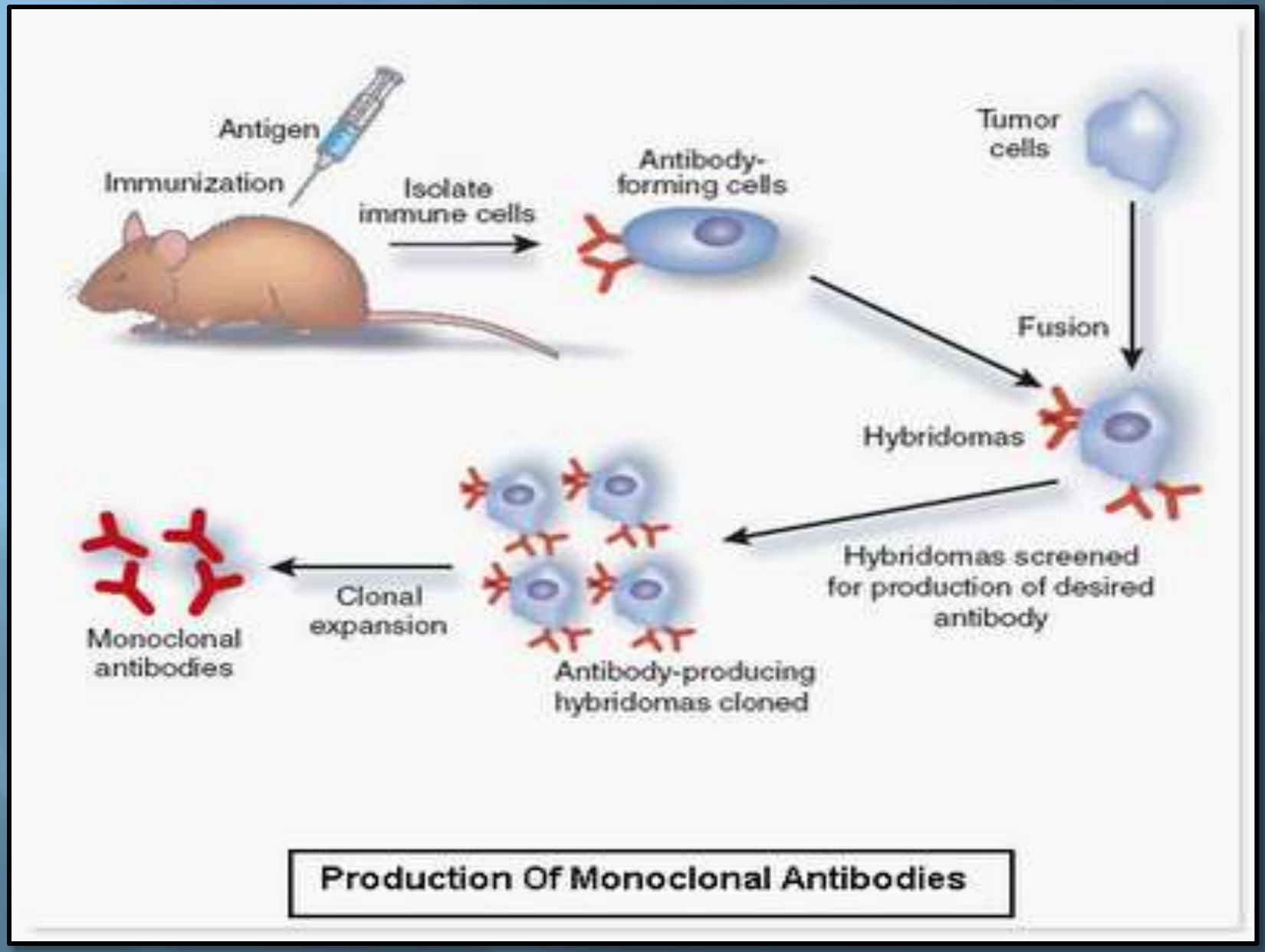



Immunization
Antibody-forming cells
Tumor cells
Tissue culture
Fusion
Hybridomas
Hybidromas screened for antibody production
Antibody-producing hybridomas cloned
Monoclonal antibodies isolated for cultivation
Monoclonal Antibody Production

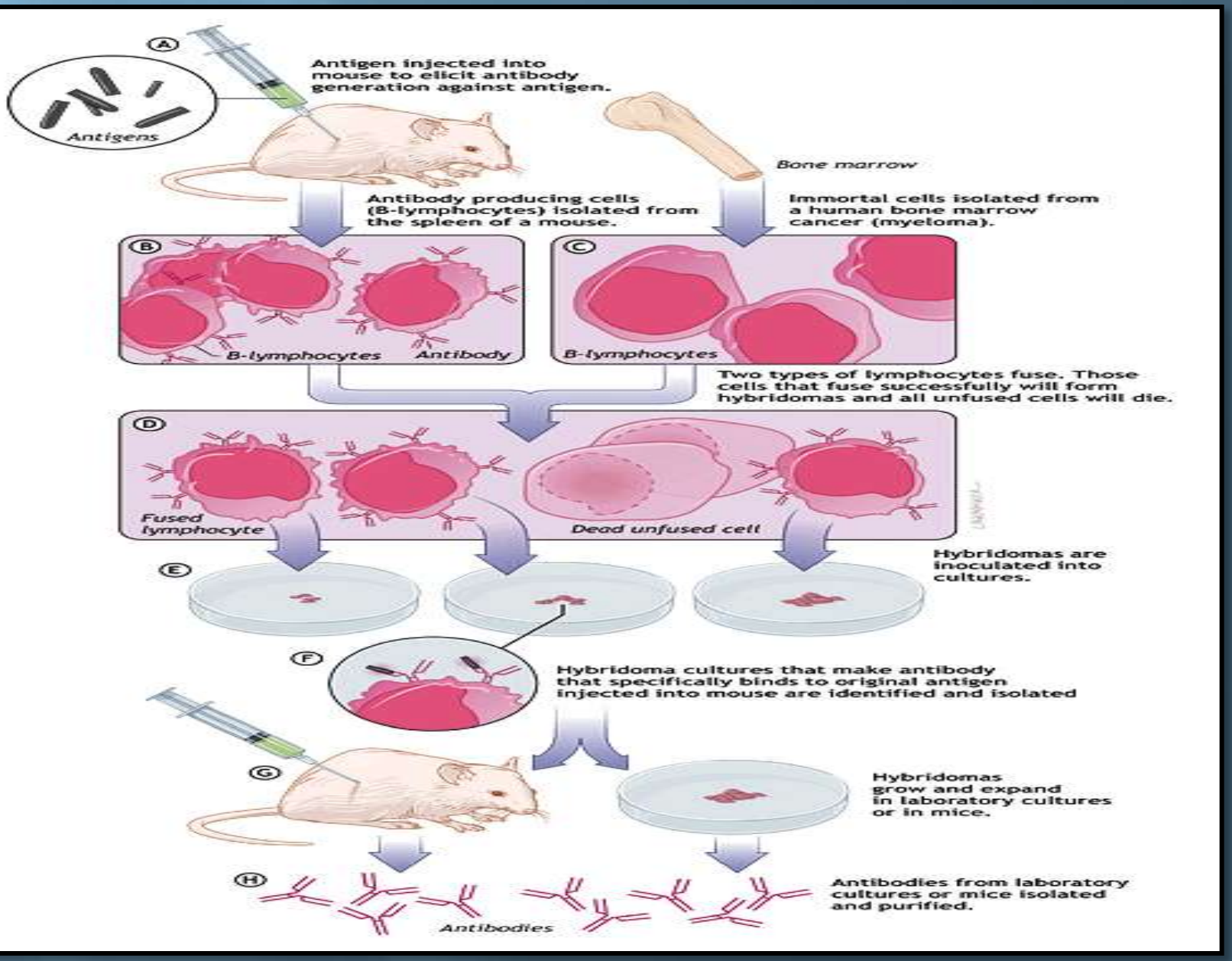
PRODUCTION OF MONOCLONAL ANTIBODIES:




Activation of lymphocytes
Culture of myeloma cell line
Mixture of cells
Fusion of cells
Selection of hybrid cells on hybridoma
Hybridoma
Separation of cells
Analysis of antibody-producing cells
Cloning of antibody-producing cells



Production Of Monoclonal Antibodies


A
Antigens
Antigen injected into mouse to elicit antibody generation against antigen.
Bone marrow
Antibody producing cells (B-lymphocytes) isolated from the spleen of a mouse.
Immortal cells isolated from a human bone marrow cancer (myeloma).
B
B-lymphocytes
Antibody
C
B-lymphocytes
Two types of lymphocytes fuse. Those cells that fuse successfully will form hybridomas and all unfused cells will die.
D
Fused lymphocyte
Dead unfused cell
Hybridomas are inoculated into cultures.
E
F
Hybridoma cultures that make antibody that specifically binds to original antigen injected into mouse are identified and isolated
G
Hybridomas grow and expand in laboratory cultures or in mice.
H
Antibodies
Antibodies from laboratory cultures or mice isolated and purified.

STEPS INVOLVED IN PRODUCTION OF MONOCLONAL ANTIBODIES

IMMUNIZATION OF MICE AND ISOLATION OF B-LYMPHOCYTES

- 2-4 weeks old mice are immunized with the known antigen by sub-cutaneous injection.
- A mouse is killed after 72 hours of immunization, especially within 4days, and its spleen is taken.
- The spleen is minced into small fragments and the fragments are sterilized.
- The fragments are then macerated into individual cells using enzymatic method.
- They are grown in fresh medium for cell fusion.

- The cell suspension so obtained is immediately suspended in a balanced salt solution.

- The suspension is washed 2 or 3 times with the balanced salt solution to get pure plasma cells (spleenocytes).

- Some of the spleenocytes are the antibody producing B-lymphocytes (B-cells).

ISOLATION OF MYELOMA CELLS

- **Myeloma cells are fast growing large cells of hematopoietic portion of bone marrow.**
- **Myeloma is taken from a bone and macerated to get a suspension of myeloma cells.**
- **The myeloma cells have the ability to produce a specific antibody in larger amount.**
- **HGPRT – mutant myeloma cells (defective in hypoxanthine guanine phosphoribosyl transferase enzyme) are raised by inducing mutations using 8-azaguanine.**
- **This will help us to select hybridoma from fused and Unfused cells.**

GENERATION OF HYBRIDOMAS BY FUSION

- The spleenocytes and myeloma cells are mixed together in the ratio of 2:5 and treated with PEG.
- The cell mix is shaken well for 3 minutes.
- The PEG brings the two cells together and induces cell fusion.
- As a result, spleenocyte myeloma hybrids called hybridomas are formed.
- Sometimes, PVA is used as a fusagen to induce the cell fusion.

SELECTION OF HYBRIDS

HAT SELECTION METHOD

- The cell suspension is diluted and centrifuged to remove the fusagen.

The cell suspension is then diluted slowly with a serum free medium containing Hypoxanthine, Aminopterin and Thymidine.

- The diluted cell suspension is distributed into the wells of a multiwell plate and the plate is incubated at 25-29^{0}C for 2-3 weeks in an incubator.
- The wells of the multiwell plate are visualized for cell clumps.

The HGPRT mutant myeloma cells fail to synthesize purines from hypoxanthine. Aminopterin blocks the metabolism of purines.

- Hybridomas synthesize purines from hypoxanthine and grow into cell clumps called clone

SCREENING OF HYBRIDS

- The wells of a multi well plate are coated with the antigen that was used to immunize the mouse.

- A small amount of medium was taken from each well having hybridoma and poured into the antigen coated well.

- Antibodies bind with epitope of the antigen and form antigen antibody complex. The unbound antibodies are washed out of the wells.

- Then a secondary antibody that specifically binds with mouse antibodies, is linked with an enzyme. The enzyme coupled antibody is poured into the wells. The secondary antibody binds with the primary antibody.

- The unbound secondary antibody is washed out. The colorless compound, on which the linked enzyme acts, is added to the wells.

- Those wells giving positive color change indicate that the corresponding wells in the original plate have hybridomas.

- Hybridoma clones in the respective wells are taken and sub-cultured in fresh medium in small flasks for week.

- The supernatants are taken from these flasks and tested separately for the amount of antibody produced. The hybridoma clone producing monoclonal antibody in higher proportion is picked up and cultured in fresh medium in a flask.

CLONING OF HYBRID CELLS

Cloning in soft agar

- In soft agar cloning, heterogenous mixture of hybrid cells is separated by localizing growth of single cells in soft agar.

- After colonies grow to sizes visible to naked eye, they can be picked up from agar with pasture pipette and then grow in fresh media.

CLONING BY LIMITED DILUTION

- In limited dilution technique, hybrid cells are diluted in such a way that 1 ml would contain 1-3 cells.
- 1/10 mm of this is added to wells.
- Thus each well theoretically would contain 0.1 to 0.3 cells.
- These are allowed to grow into colonies.
- These clones of hybrid cells can then be grown in large volumes to obtain monoclonal antibodies

PRODUCTION OF MONOCLONAL ANTIBODIES

In vivo method

•The hybridoma cell line that makes a desired monoclonal antibody is injected into the body of mice through intra-muscular injection.

• The mice are grown in the laboratory for 3 or 4 weeks.

•Their ascetic fluid or blood is taken and the monoclonal antibodies are isolated from it.

•By this method, a mouse can produce about 50 mg of MCA.

SUSPENDED CELL CULTURE IN FERMENTERS

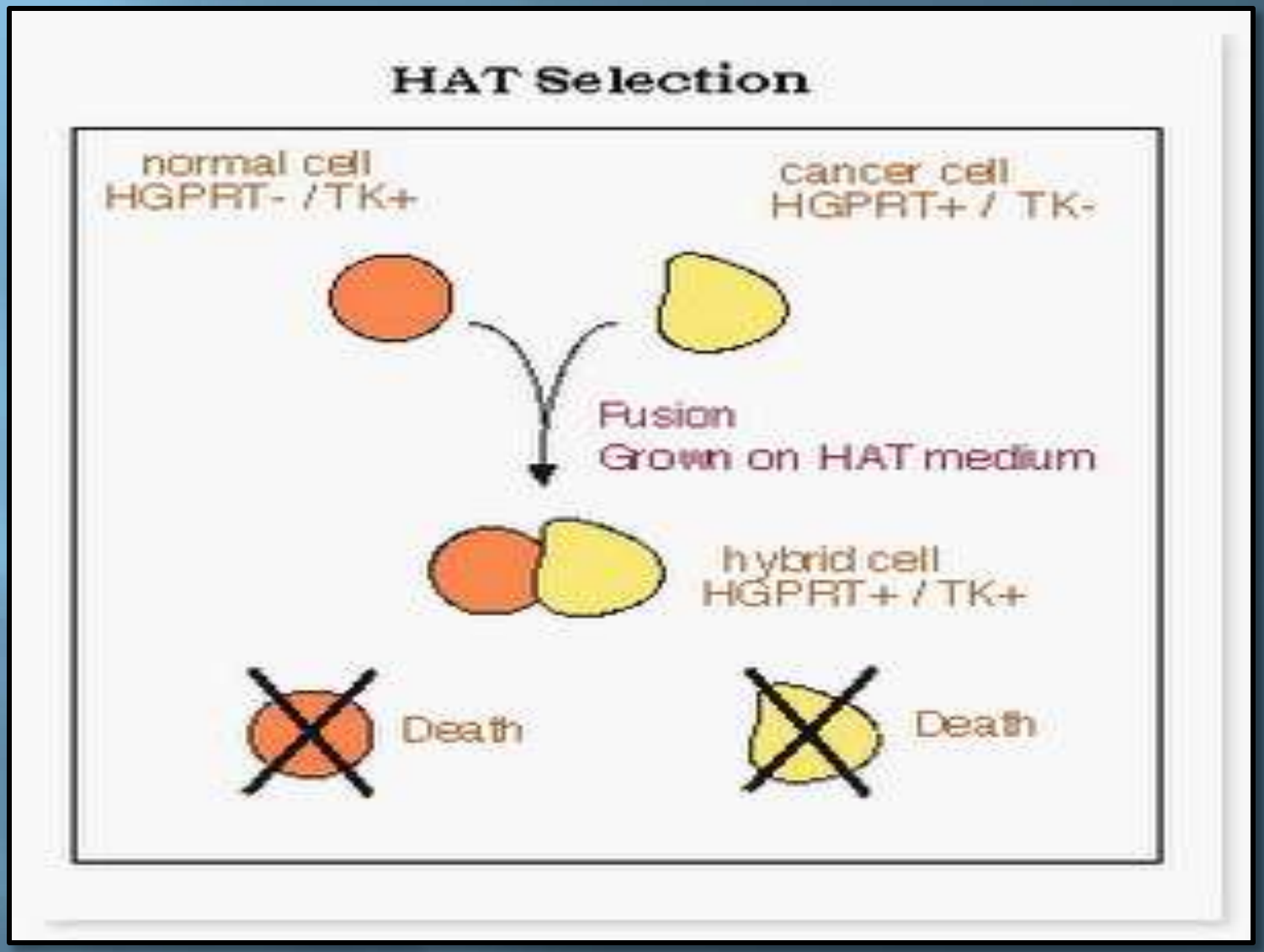
•A hybridoma clone is cultured in a large fermenter using chemically defined complex medium having all minerals, vitamins and co-factors. Airlift fermenter is of much use for this purpose.

•In this method, one liter of culture broth can yield 100 mg of MCA in 2 weeks.

IMMOBILIZED CELL REACTORS

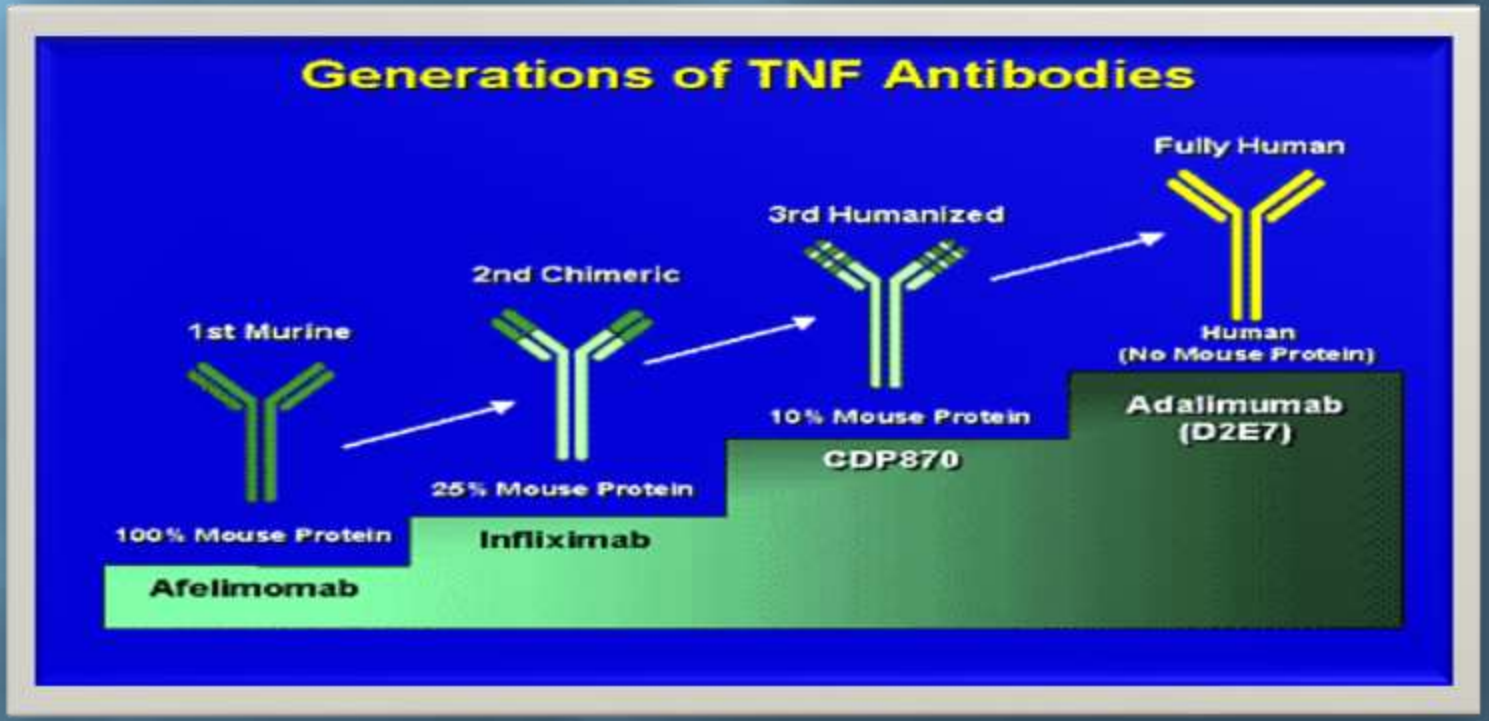
•Cells of hybridoma clones are immobilized in a hollow fiber reactor using polyacrylamide gel.

• By this method, a few grams of MCA can be produced within 2 weeks.


HAT Selection
normal cell
HGPRT- / TK+
cancer cell
HGPRT+ / TK-
Fusion
Grown on HAT medium
hybrid cell
HGPRT+ / TK+
Death
Death

TYPES OF MONOCLONAL ANTIBODIES

- Murine Monoclonal Antibodies
- Chimeric Monoclonal Antibodies
- Humanized Monoclonal Antibodies
- Human Monoclonal Antibodies



APPLICATIONS OF MONOCLONAL ANTIBODIES

- Diagnosing diseases such as cancer or infectious diseases.
- Monoclonals are also used as therapeutic molecules.
- Biological Reagents in diversified discipines.
- **Miscellaneous Applications**
  - ✓Drug delivery and targeting
  - ✓Detection of surface molecules.
  - ✓ Detection of Antigens in blood.
  - ✓ Detection of drugs in urine.
  - ✓ For reduction of organ transplant rejections.

ADVANTAGES OF MONOCLONAL ANTIBODIES

- Pure one molecular species only.
- Specificity for one antigenic determinant.
- Monoclonals recognize only one epitope of the antigen and are highly specific to that particular antigen, and thus will usually give substantially less background staining and the homogeneity of monoclonal antibodies is very high.
- *In vitro* and *In vivo* production is possible with high production rate.
- Immortal cell lines.
- If experimental conditions are kept constant, reproducibility of results is very high.
- Monoclonal antibodies have the advantage of being available in unlimited quantities.
- Being a homogeneous product, they do not need purification.

LIMITATIONS OF MONOCLONAL ANTIBODIES

- Cancer cells are heterogeneous, so those cells that are not recognized by the monoclonal antibody can escape.
- Some tumors contain semisolid cores with poor circulation and thus can not be reached by monoclonals.
- Radio immuno conjugates are monoclonal antibodies to which radio nuclides have been conjugated to provide cytotoxic radiation after the monoclonal antibodies binds to its target antigen.
- The isotopes must commonly used are Iodine-131 and Yttrium-90, both of which are B-emitters.
- The high specificity of monoclonal antibodies may pose problems in the detection of viruses with extensive antigenic variation, such as influenza A

PRINCIPLES OF TARGETING

- To deliver the drug in a manner such that it is preferentially localized at desired site of action is called as **TARGETING**.
- ***Active Targeting***

  The natural disposition pattern of a carrier is modified to target it to specific organs, tissues or cells.

- Monoclonal antibodies would thus appear to be the more generally applicable mode of active targeting.

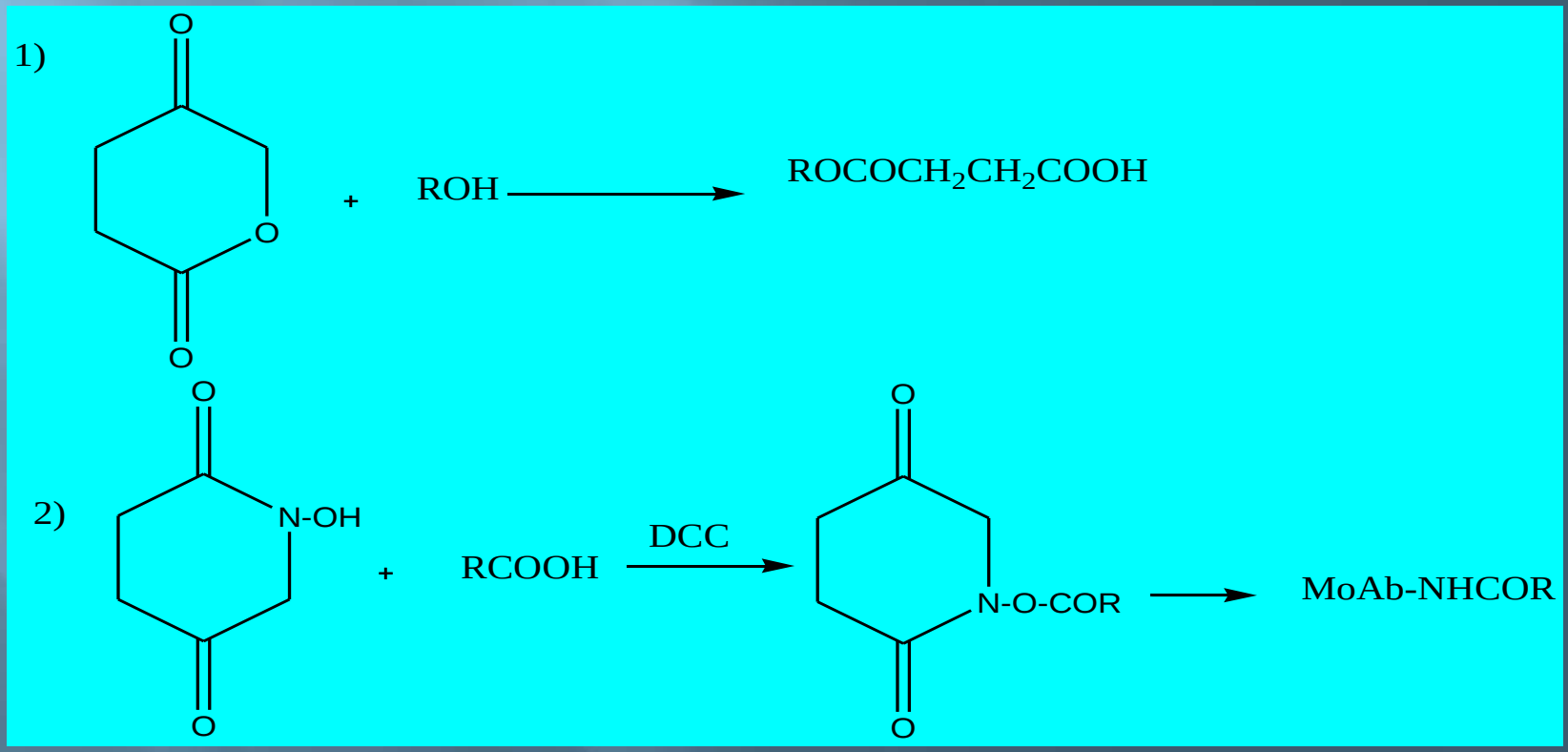
IDEAL CHARACTERISTICS OF ANTIBODY DIRECTED DRUG DELIVERY SYSTEM

- The produced antibody should have high efficacy.
- It should be stable upon conjugation with drug, both under shelf storage conditions and in the circulation after injection.
- It should restore the antigen-binding ability after conjugated with drug.
- After reaching the target site, it should have the desired pharmacological effect equivalent to free drug or must release free drug or a derivative that is fully efficacious.

- An important part of the design of an antibody directed drug delivery system is the type of linkage and coupling method between anti body and drug.
  - The drug can be covalently bound to the monoclonal antibody directly and can be conjugated through a linker such as water soluble polymer.
  - A carrier such as a liposome of a polymeric micro sphere can be used wherein the drug is entrapped in or bound to the carrier and monoclonal antibody is bound to the surface of the carrier.
  - Amino, sulphahydryl and carboxylic groups are the most common functional groups on the antibody carrier and drug used for coupling.

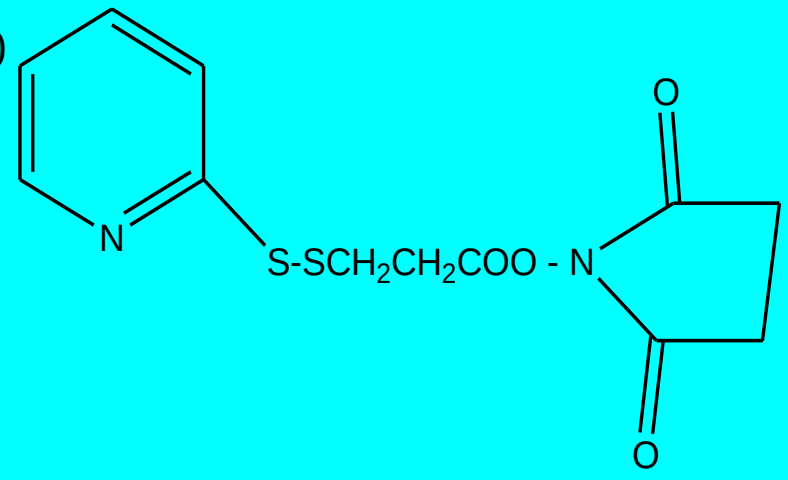
For example:

- Succinic anhydride can convert an alcohol or amino group to carboxylic group.
- For linkage of drug to antibody classical protein cross linking reagents have been used to prepare immuno conjugates for ex: carbodiimide reagents link amino group with carboxylic group via amide bond.



Other examples of conjugation of monoclonal antibody with drugs

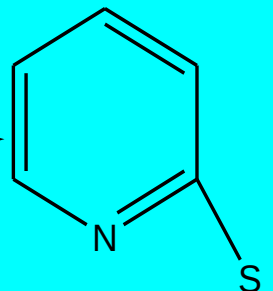
3)



n-succimidyl-3-(pyriddyl dithio)propionate

\+

RNH_2



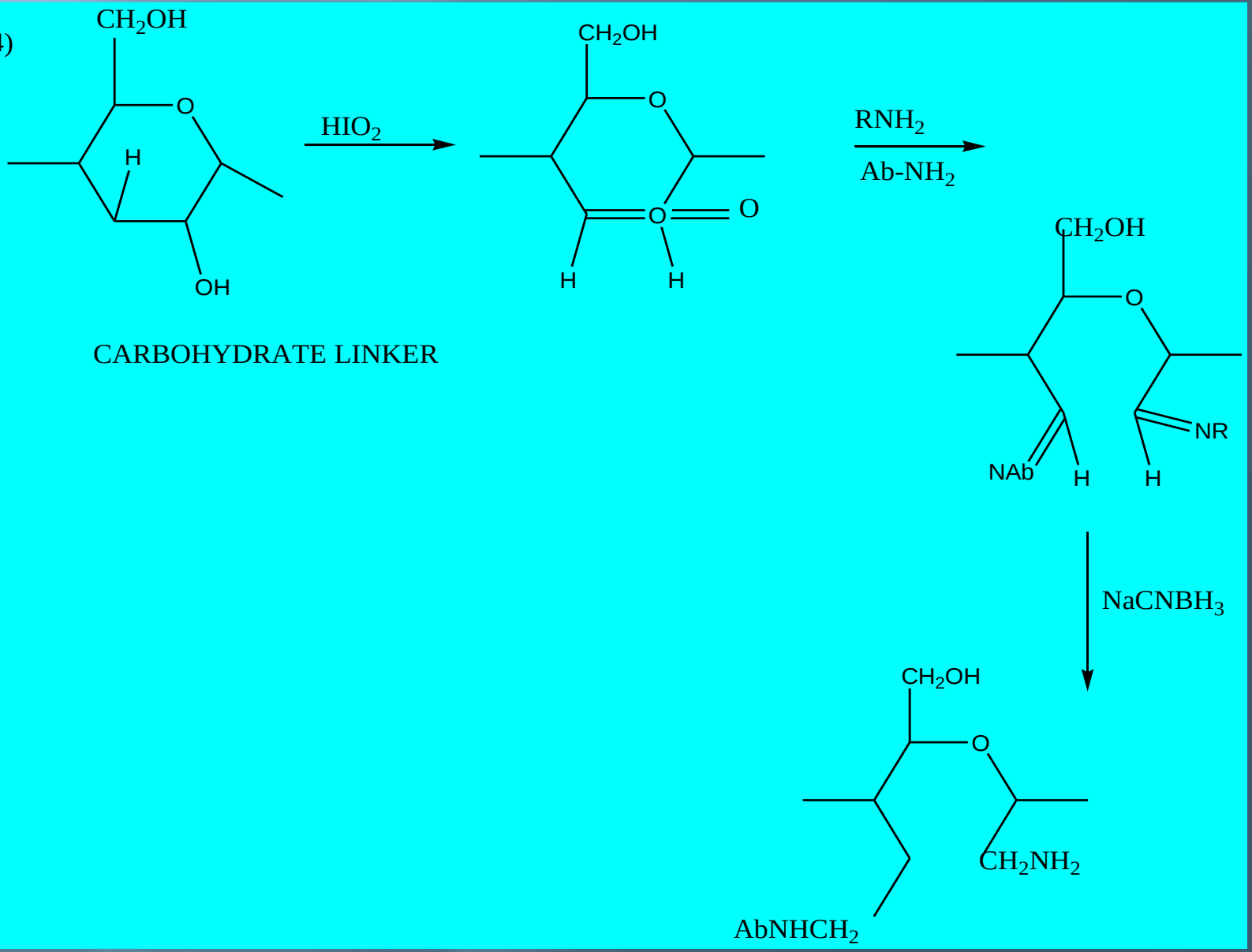
RNHCOH2CH2C S

dithiothrttol

MOAb-SH

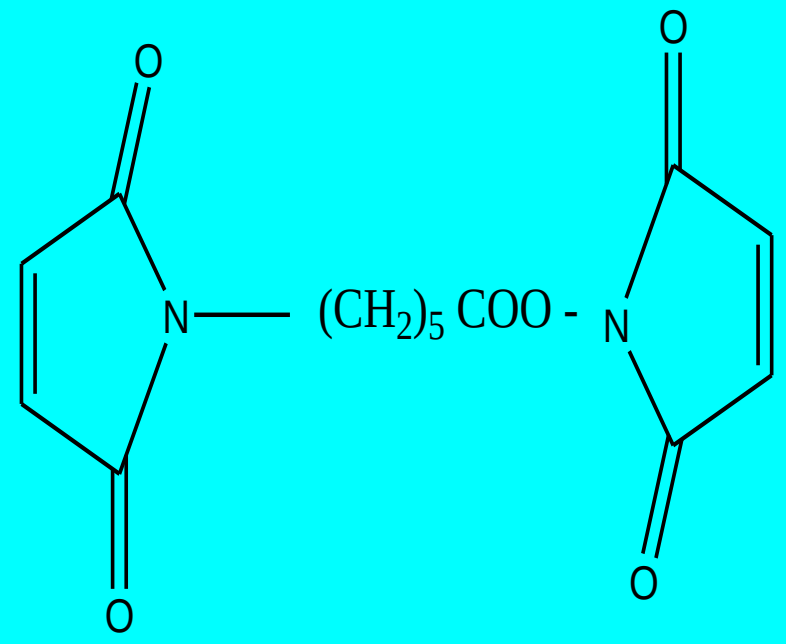
MOAb-S-SCH_2-CH_2-CO NHR

4)



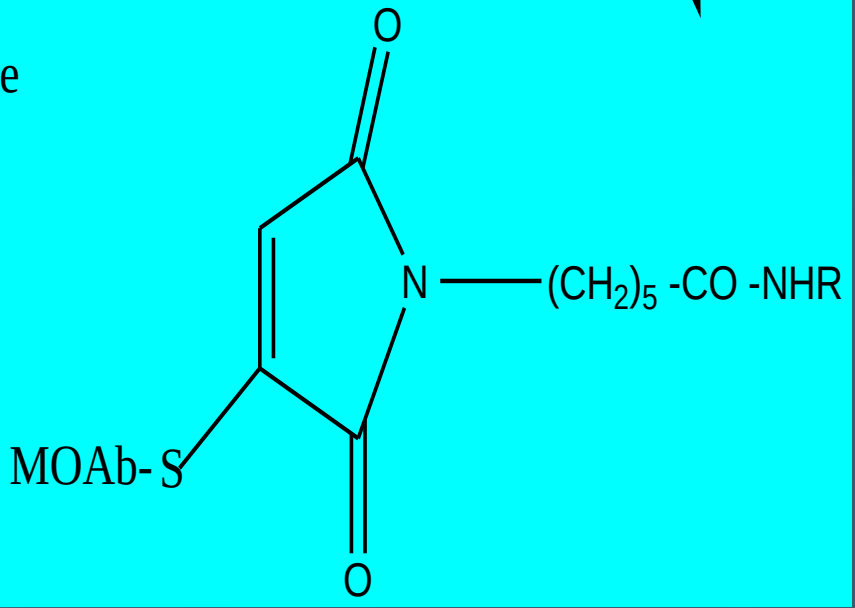
CARBOHYDRATE LINKER

5)



+ MOAb-SH + RNH_2

N-[-6maleicidocarbonyloxyl]succinimide



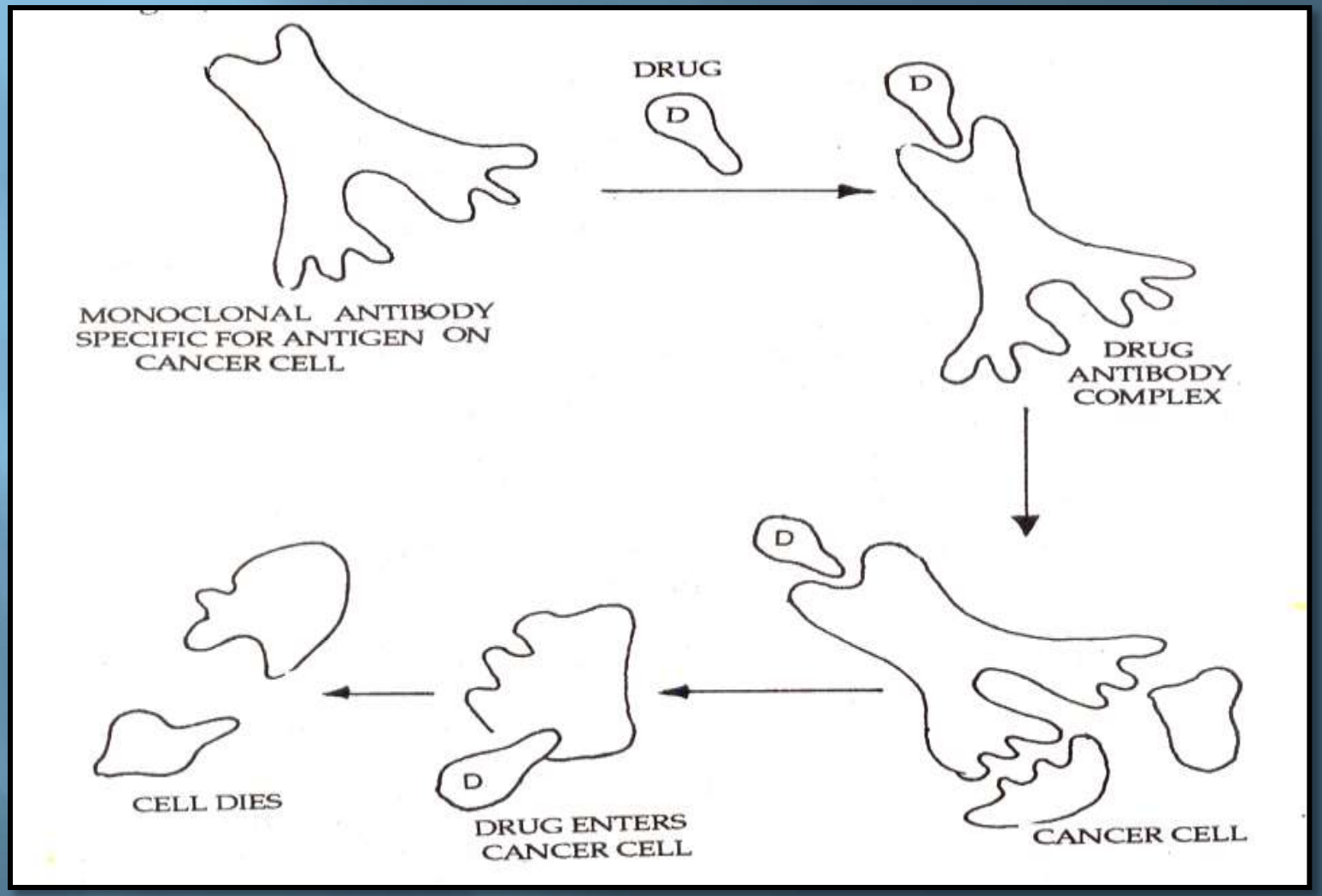
IMMUNOTHERAPY WITH UNCONJUGATED MONOCLONAL ANTIBODIES IN CANCER TREATMENT

- Unconjugated monoclonal antibodies are able to kill caner cells by several mechanisms initiated that are responsible for therapeutic effect.
- One is antibody dependent cellular toxicity (ADCT)
- The other is complement dependent cytotoxicity (CDC)

❖Monoclonal antibody binding to the target antigens on cell surfaces can also acts as “blocking” antibodies, interfering with the binding of certain peptides or growth factor needed for cell growth.

- Immunoconjugates of chlorombucil have shown improved therapeutic activity.
- Bispecific monoclonal antibody composed of anti-CD3 of anti-CD2 monoclonal antibody, chemically conjugated to antitumor antibody and coated on lymph line- activated killer (LAK) cells, have been clinically investigated for the treatment of malignant glaucoma, lymphoma and ovarian cancer with encouraging results.
- In malignant glaucoma therapy, bispecific monoclonal antibody-coated LAK cells were injected intracranially following surgical removal of tumor and whole brain irradiation and or chemotherapy.
- This resulted in 76% of patients being tumor-free.

CONJUGATED MONOCLONAL ANTIBODIES IN CANCER TREATMENT



RADIO IMMUNO CONJUGATES IN CANCER TREATMENT

- A number of antitumor agents, including *chlorambucil methotrexate, daunomycin, and doxorubicin*, conjugated to tumor specific antibodies in tumor (Cancer) drug delivery.
- The most extensively studied has been a *doxorubicin- BR 96 immunoconjugate*.
- BR 96 is a chimeric monoclonal antibody specific for Lewis antigen found on the surface of tumor cell.

- The immunoconjugate is formed using acid – labile hydrazone linkage attached through the thio groups of the monoclonal antibody, with 8 moles of doxorubicin/mole of monoclonal antibody.
- After rapid internalization into antigen-bearing cells, the conjugate is designed to release free doxorubicin from the monoclonal antibody hydrazone linkage in the acidic environment of the lysosome.
- Another immunoconjugate is CMA -676, which is a conjugate of an anti – CD33 monoclonal antibody and calcheamicin, an anticancer drug shown to be 1000-fold more potent than doxorubicin in animal models.

TARGETED DELIVERY OF RADIO LABELLED ANTIMYOSIN ANTIBODY IN ACUTE MYOCARDIAL INFRACTION

- The cell membranes of normal cardiac cells are composed of the same lipid bilayer as that found on necrotic cells.
- The cardiac myofilaments of normal cells are not exposed to the extra cellular environment. But those of necrotic cells are exposed the extra cellular milieu.
- Therefore such structures provide new targets for delineation of the necrotic from non necrotic myocardium.

- Anti cardiac myosin antibody was chosen as the targeting moiety for the delivery of drug for diagnostic imaging of acute myocardial infarction.
- Hypoxic neonatal murine myocytes are treated with antimyosin antibody and are attached covalently to 1 μm diameter polystyrene beeds.
- These are targeted for diagnosis of myocardial infarction.

ANTIMYOSIN IN DIAGNOSIS OF VARIOUS CARDIOMYOPATHIES (MYOCARDITIS)

- Cardiomyopathy is disease of an abnormal condition of myocardium.
- Myocarditis is a cardiomyopathy of highly variable clinical manifestations that can lead to dilated cardiomyopathy and heart failure.
- It is believed to have a viral origin but the chronic component of the etiology is believed to be autoimmune in nature.
- For the diagnosis of myocarditis, the antimyosin immunoscintigraphy is able to target the myonecrotic component of the disease and provide a very sensitive diagnostic indicator non invasive diagnosis of myocarditis.

THERAPEUTIC MONOCLONAL ANTIBODY DRUGS CURRENTLY MARKETED

GENERIC NAME	TRADE NAME (COMPANY)	TYPE OF MoAb	APPLICATION(S)
Rituximab	Rituxan (IDE/ Genentech)	Chimeric anti-CD20	Non-Hodgkins Lymphoma
Trasuzumab	Herceptin (Genentech)	Humanized anti HER2	Matastatic breast cancer
Palivizumab	Synagis (Medimmune)	Humanized anti-RSV epitope	Antiviral (Respiratory tract disease)
Muromonab – CD3	Orthoclone (OKT3)	Murine anti-CD3	Immuno suppressant
Abciximab	Reopro (Centocor)	Fab fragment of chimeric anti – 7E3	Platelet aggregation inhibitor

LIST OF MONOCLONAL ANTIBODIES, WHICH ARE CURRENTLY IN CLINICAL TRAIL

S.No	Indication	Antibody Name	Sponsors	Trail Status
1	Anticoagulant	Corsevin M	Centocor	1
2	Asthma	Rhu MAb – E25	Genetech	3
3	Autoimmune disease	Smart anti CD3	Protein design lab	2
4	Cancer	Anti – VEGF	Genetech	3
5	Ovarian	OvaRex	Altarex	3
6	Lung	BEC2	Merck-Lga A	Approved
7	Crohn's Disease	Infliximab	Centocor	FDA Approved
8	Myocardial infarction	Anti-CD18	Genetech	2
9	Sarcoma	Vitaxam	Med immune	2
10	HIV	PRO 542	Progeneics/Genzyme	2
11	Hep B	Ostovir	Transgenics	2

THANK YOU