MONOCLONAL ANTIBODIES: Preparation, Evaluation & Application

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Basic concepts and Introduction

MONOCLONAL ANTIBODIES
What are antibodies?

• An antibody is a **protein used by immune system to identify and neutralize foreign objects** like bacteria and viruses. Each antibody recognizes a specific antigen unique to its target.

• The **high specificity of antibodies** makes them an excellent tool for detecting and quantifying a broad array of targets, from drugs to serum proteins to microorganisms.

• With **in vitro assays**, antibodies can be used to **precipitate soluble antigens, agglutinate (clump) cells, opsonize and kill bacteria** with the assistance of complement, and neutralize drugs, toxins, and viruses.
An antibody binds to a specific region on an antigen called an epitope. A single antigen can have multiple epitopes for different, specific antibodies.
Monoclonal antibodies

- Monoclonal antibodies are identical immunoglobulins, generated from a single B-cell clone. These antibodies recognize unique epitopes, or binding sites, on a single antigen. Derivation from a single B-cell clones and subsequent targeting of a single epitope is what differentiates monoclonal antibodies from polyclonal antibodies.

- Polyclonal antibodies are antibodies that are derived from different cell lines. They differ in amino acid sequences.
Characters of monoclonal Antibodies

• Monoclonal antibodies (mAB) are single type of antibody that are identical and are directed against a specific epitope (antigen, antigenic determinant) and are produced by B-cell clones of a single parent or a single hybridoma cell line.

• A hybridoma cell line is formed by the fusion of one B-cell lymphocyte with a myeloma cell.

• Some myeloma cell synthesize single mAB antibodies naturally.
|                          | Polyclonal antibodies                                           | Monoclonal antibodies                              |
|--------------------------|-----------------------------------------------------------------|----------------------------------------------------|
| Produced by:             | Many B cell clones                                               | A single B cell clone                              |
| Binds to:                | Multible epitopes of all antigen used in the immunization       | A single epitope of a single antigen                |
| Antibody class:          | A mixture of different Ab classes (isotypes)                     | All of a single Ab class                           |
| Ag-binding sites:        | A mixture of Abs with different antigen-binding sites            | All Abs have the same antigen binding sites        |
| Potential for cross-reactivity: | High                                                             | Low                                               |

Differences between polyclonal and Monoclonal antibodies
Advantages of using Monoclonal Antibodies:

• Though expensive, monoclonal antibodies are cheaper to develop than conventional drugs because it is based on tested technology.

• Side effects can be treated and reduced by using mice-human hybrid cells or by using fractions of antibodies.

• They bind to specific diseased or damaged cells needing treatment.

• They treat a wide range of conditions.
Disadvantages of using Monoclonal Antibodies:

- **Time consuming project** - anywhere between 6 - 9 months.
- **Very expensive** and needs considerable effort to produce them.
- Small peptide and fragment antigens may not be good antigens - monoclonal antibody **may not recognize the original antigen**.
- Hybridoma culture may be subject to **contamination**.
- System is **only well developed for limited animal** and not for other animals.
- More than **99% of the cells do not survive** during the fusion process – reducing the range of useful antibodies that can be produced against an antigen.
- **It is possibility of generating immunogenicity.**
History and Development

MONOCLONAL ANTIBODIES
Monoclonal Antibodies: History and Development

- **Paul Enrlich** at the beginning of 20th century coined the term “magic bullets” and postulated that, if a compound could be made that selectively targets a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity.

- In the 1970s, the **B-cell cancer multiple myeloma** was known. It was understood that these cancerous B-cells all produce a single type of antibody (a paraprotein).
In 1975, Kohler and Milstein provided the most outstanding proof of the clonal selection theory by fusion of normal and malignant cells (Hybridoma technology) for which they received Nobel prize in 1984.

In 1986, first monoclonal antibody was licenced by FDA. Orthoclone OKT3 (muromonab-CD3) which was approved for use in preventing kidney transplant rejection.
Preparation

MONOCLONAL ANTIBODIES
Preparation of Monoclonal Antibodies

- Monoclonal Antibody production or mAb is produced by cell lines or clones obtained from the immunized animals with the substances to be studied. Cell lines are produced by fusing B cells from the immunized animal with myeloma cells.

- To produce the desired mAB, the cells must be grown in either of two ways: by injection into the peritoneal cavity of a suitably prepared mouse (in vivo method) or by in vitro tissue culture.

- The vitro tissue culture is the method used when the cells are placed in culture outside the mouse’s body in flask.
Preparation of Monoclonal Antibodies

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Practical steps for production

1. Immunize animal
2. Isolate spleen cells (containing antibody-producing B cell)
3. Fuse spleen cells with myeloma cell (using PEG)
4. Allow unfused B cell to die
5. Add aminopterin to culture and kill unfused myeloma cells
6. Clone remaining cells (place 1 cell/well and allow each cell to grow into a clones of cell)
7. Screen supernatant of each clone for presence of desired antibody
8. Grow chosen clone of cells in tissue culture indefinitely
9. Harvest antibody from the culture.
10. $1000-2000 per mg
1. Mouse is immunized with antigen X, and mouse spleen produces plasma cells that secrete antibodies against the antigen.

2. Myeloma cells unable to produce antibodies or HGPRT are selected.

3. Mouse spleen is removed. Plasma cells from spleen are isolated and mixed with myeloma cells. Cell fusion is induced to produce hybridomas.

4. Cells are transferred to HAT medium.

5. Hybridomas that produce antibodies specific to antigen X are selected and grown in bulk.
Evaluation

MONOCLONAL ANTIBODIES
Evaluation

• “Guidelines on evaluation of monoclonal antibodies as similar biotherapeutic products”, WHO, 1st March, 2016

• “Guideline on development, production, characterisation and specification for monoclonal antibodies and related products”, EMA, 21st July, 2016
Process and Evaluation
1. Characterisation of monoclonal antibodies

- Physicochemical characterisation
- Immunological properties
- Biological activity
- Purity, impurity and contaminants
- Quantity
2. Specifications

• Identity
• Purity and impurities
• Potency
• Quantity
• General tests
1.1 Physicochemical characterisation

• A physicochemical characterisation program will generally include a determination of the class, subclass, light chain composition and primary structure of the monoclonal antibody.

• The class or subclass of an antibody is defined by its heavy chain. There are five main classes of antibodies: M, G, A, E, and D. The method of antibody purification will differ based on the class.

• The amino acid sequence should be deduced from DNA sequencing and confirmed experimentally by appropriate methods (e.g. peptide mapping, amino acid sequencing, mass spectrometry analysis).

• The variability of N- and C-terminal amino-acid sequences should be analysed (e.g. C-terminal lysine(s)).
Classes based on constant region of heavy chains

- Immunoglobulin A (IgA)
- Immunoglobulin D (IgD)
- Immunoglobulin E (IgE)
- Immunoglobulin G (IgG)
- Immunoglobulin M (IgM)

Differentiation of heavy chains

- Length of C region, location of disulfide bonds, hinge region, distribution of carbohydrate

Types of Immunoglobulin
1.2 Immunological properties

• Binding assays of the antibody to purified antigens and defined regions of antigens should be performed, where feasible, to determine affinity, avidity and immunoreactivity (including cross reactivity with other structurally homologous proteins).

• Unintentional reactivity/cytotoxicity for human tissues distinct from the intended target should be documented.

• The epitope and molecule bearing the relevant epitope should be defined. This should include a biochemical identification of these structures (e.g. protein, oligosaccharide, glycoprotein, glycolipid), and relevant characterisation studies (amino acid sequence, carbohydrate structure) to the extent possible.
Immunogenicity potential based on source of Ig

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1.3 Biological activity

- The biological activity (i.e. the specific ability or capacity of a product to achieve a defined biological effect) should be assessed by appropriate *in vitro* assay(s).
- Where *in vivo* assays are necessary, the use of such assays should be thoroughly justified.
- The mechanism of action and the importance (or consequences) of the product effector functions with regards to the safety and efficacy of the product should be discussed.
1.4 Purity, impurity and contaminants

- These methods generally include the determination of physicochemical properties such as molecular weight or size, extinction coefficient, electrophoretic profiles, chromatographic data and spectroscopic profiles.

- Potential process-related impurities (e.g. HCP, host cell DNA, cell culture residues, downstream processing residues) should be identified, and evaluated qualitatively and/or quantitatively, using chromatographic technique.

- Contaminants, which include all adventitiously introduced materials not intended to be part of the manufacturing process (e.g. microbial species, endotoxins) should be strictly avoided and/or suitably controlled.
1.5 Quality

• Quantity should be determined using an physicochemical and/or immunochemical assay.

• It should be demonstrated that the quantity values obtained are directly related to those derived using the biological assay.

• When this correlation exists, it may be appropriate to use measurement of quantity rather than the measurement of biological activity in the product labelling and manufacturing processes, such as filling.
2. Specifications

- Specifications are one part of a total control strategy designed to ensure product quality and consistency, and when tested, the product should be in compliance with its specification.
- Specifications should be set and take into account relevant quality attributes identified in characterisation studies.
- Selection of tests to be included in the specifications is product specific.
- The rationale used to establish the acceptable range of acceptance criteria should be described.
2.1 Identity

• The identity test(s) should be highly specific and should be based on unique aspects of the product’s molecular structure and/or other specific properties (e.g. peptide map, anti-idiotype immunoassay, or other appropriate method).

• Considering the great similarity of the constant domains of different antibodies, more than one test (physicochemical, biological and/or immunochemical) may be necessary to establish identity, and such test(s) should be able to discriminate other antibodies that may be manufactured in the same facility.
2.2 Purity and Impurities

- As noted in the characterisation section, monoclonal antibodies may display a complex purity/impurity profile that should be assessed by a combination of orthogonal methods, and for which individual and/or collective acceptance criteria should be established for relevant product-related variants.

- For example, separation methods based on charge heterogeneity is considered to quantitatively and qualitatively monitor charge variants.

- Considering that glycosylation may have an impact on the pharmacokinetics of the product, and may modulate its immunogenic properties, appropriate acceptance criteria should be considered for this attribute. In addition, such control will further confirm the consistency of the product.
2.3 Potency

- Potency is the quantitative measure of biological activity based on an attribute of the product which is linked to the relevant biological properties. A relevant potency assay should be part of the specifications for drug substance and/or drug product, and should ideally reflect the biological activity in the clinical situation.

- For antibodies for which the clinical activity is only dependent on binding/neutralising properties, a potency assay that measures binding to the target (i.e. binding assay) is acceptable when appropriately justified.
2.4 Quantity

- The quantity of the drug substance, usually based on protein content (mass), is determined chromatographically using reference standard.

2.5 General tests

- Appearance, solubility, pH, osmolality, extractable volume, sterility, bacterial endotoxins, stabiliser and water, is assessed where appropriate.
Applications

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Major Applications:

(1) **Diagnostic Applications**
- Biochemical analysis
- Diagnostic Imaging

(2) **Therapeutic Applications**
- Direct use of MAbs as therapeutic agents
- MAbs as targeting agents.

(3) **Protein Purification**
1a. Biochemical analysis

- Routinely used in radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) in the laboratory.
- These assays measure the circulating concentrations of hormones (insulin, human chorionic gonadotropin, growth hormone, progesterone, thyroxine, triiodothyronine, thyroid stimulating hormone) and several other tissue and cell products (blood group antigens, blood clotting factors, interferon’s, interleukins, tumor markers).

Eg. Pregnancy by detecting the urinary levels of human chorionic gonadotropin.

- Hormonal disorders analysis of thyroxine, triiodothyronine.
- Cancers estimation of plasma carcinoembryonic antigen in colorectal cancer, and prostate specific antigen for prostate cancer.
1b. Diagnostic imaging

- **Radiolabeled**—MAbs are used in the diagnostic imaging of diseases, and this technique is referred to as immunoscintigraphy. The radioisotopes commonly used for labeling MAb are iodine—131 and technetium—99. The MAb tagged with radioisotope are injected intravenously into the patients.

- These MAbs localize at specific sites (say a tumor) which can be detected by imaging the radioactivity. In recent years, single photon emission computed tomography (SPECT) cameras are used to give a more sensitive three dimensional appearance of the spots localized by radiolabeled—MAbs.

- Myocardial infarction, DVT, atherosclorosis etc.
2a. Direct use of MAbs as therapeutic agents

- In destroying disease-causing organisms: MAbs promote efficient opsonization of pathogenic organisms (by coating with antibody) and enhance phagocytosis.

- In the immunosuppression of organ transplantation: In the normal medical practice, immunosuppressive drugs such as cyclosporin and prednisone are administered to overcome the rejection of organ transplantation. In recent years, MAbs specific to T-lymphocyte surface antigens are being used for this purpose.
• **In the treatment of cancer:** MAbs, against the antigens on the surface of cancer cells, are useful for the treatment of cancer. The antibodies bind to the cancer cells and destroy them via different pathways.
**In the treatment of AIDS:** Genetic engineers have been successful to attach Fc portion of mouse monoclonal antibody to human CD$_4$ molecule. This complex has high affinity to bind to membrane glycoprotein gp$_{120}$ of virus infected cells. The Fc fragment induces cell-mediated destruction of HIV infected cells.
2a. MAbs as targeting agents.

- The drugs can be coupled with MAb (directed against a cell surface antigen of the cells, say a tumor) and specifically targeted to reach the site of action.

Eg. **Alkaline phosphatase** for the conversion of phosphate pro-drugs.

**Carboxy peptidase** for converting inactive carboxyl pro-drugs to active drugs.

**Lactamase** for hydrolyzing β-lactam ring containing antibiotics.
• **MAbs in the dissolution of blood clots:**

**Fibrin** is the major constituent of blood clot which gets dissolved by plasmin. **Plasmin** in turn is formed by the activation of plasminogen by plasminogen activator.

Tissue plasminogen activator (tPA) can be used as a therapeutic agent to remove the blood clots.
Drug delivery through liposomes coupled to tissue-specific MAbs:

- Liposomes are sacs or vesicles formed spontaneously when certain lipid molecules are exposed to aqueous environment.
- Drug entrapped in liposomes that are coated with MAbs directed against tissue-specific antigens are being tried for drug delivery.
- Unfortunately, the progress in this approach has been limited, since such liposomes do not reach the target cells.
- They are retained mostly in the liver and spleen (reticuloendothelial cells), and degraded.
3. Protein Purification

- Monoclonal antibodies can be produced for any protein. And the so **produced MAb can be conveniently used for the purification of the protein against which it was raised.**
- MAbs columns can be prepared by coupling them to cyanogen bromide activated Sepharose (chromatographic matrix). The immobilized MAbs in this manner are very useful for the purification of proteins by immunoaffinity method.
- There are certain advantages of using MAbs for protein purification. These include the specificity of the MAb to bind to the desired protein, very efficient elution from the chromatographic column and high degree of purification.
Current scenario and Future perspective

MONOCLONAL ANTIBODIES
Growth of Monoclonal Antibody Market

Vast growing number of approved product in the market will increase the incidence of monoclonal market in the coming future.

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Status of monoclonal antibody products in pharmaceutical market

1. Humira (adalimumab) by AbbVie - 12,890 million USD global sale (2014)
2. Enbrel (etanercept) by Amgen - 8,915 million USD global sale (2014)
3. Remicade by Johnson & Johnson - 8,807 million USD global sale (2014)
4. Rituxan (rituximab) by Roche - 7,547 million USD global sale (2014)
5. Avastin by Roche - 7,018 million USD global sale (2014)
6. Herceptin (trastuzumab) by Roche - 6,863 million USD global sale (2014)

1/19/2017
National Institute of Pharmaceutical Education and Research, S.A.S. Nagar
• About 75 monoclonal antibodies are currently approved by the FDA for use in humans for treating various diseases and conditions including: cancer, chronic inflammatory diseases, transplantation, infectious diseases and cardiovascular diseases.

• **Glenmark**, which is seeking permission from MHRA, U.K. for conducting Phase I clinical studies for one of its mAb candidate, **GBR 900** mAb targeting TrkA, the receptor of nerve growth factor to tackle chronic pain.

• India is a fertile land for mAb market due to Large patient base, growing economy, abundant manpower and low R&D cost.
Few Commercially available mAb approved by FDA

| Name             | Trade name | Target                  | Use                                                                 |
|------------------|------------|-------------------------|----------------------------------------------------------------------|
| Abciximab        | ReoPro     | CD41 (integrin alpha-IIb) | Platelet aggregation Inhibitor                                      |
| Adalimubab       | Humira     | TNF-\textit{alpha}      | Rheumatoid arthritis, Crohn’s Disease, Plaque psoriasis, psoriatic Arthritis |
| Alirocumab       | Praluent   | PCSK9                   | Hypercholesterolemia                                                |
| Avilumab         | Bavencio   | PD-L1                   | Cancer                                                              |
| Benralizumab     | Facenra    | CD125                   | Asthma                                                              |
| Daclizumab       | Zenapax    | CD25                    | Organ transplant rejection                                          |
| Daratumubab      | Darzalex   | CD-38                   | Multiple Myeloma                                                    |

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Thank you