BHARATHIDASAN UNIVERSITY

Tiruchirappalli- 620024,
Tamil Nadu, India

Programme: M.Sc., Biomedical Science

Course Code: 18BMS59C17
Course Title: Immune & Molecular Diagnostics

Unit-I

Basic Immunological Methods

Dr. R. POORNIMA
Guest Faculty
Department of Biomedical Science

Unit I:

Basic Immunological Methods – Preparation of antigens, rising of antisera, routes of administration, doses for administration, purification of antibodies- methodology – IgG & IgA. Monoclonal antisera raising & Hybridoma technology. Types of conjugated antibodies, types of substrates and color detectors used in immunoassays. Immunofluorescence, Flow cytometry - clinical focus – Leukemia typing (immunophenotyping), ELISA and its variants- Principle and application, Surface Plasmon Resonance- principle and application- western blotting- principle and application

PRESENTATION: 1

PREPARATIONS OF ANTIGENS

1. Selection of Source Material:

The first step involves selecting the appropriate source material. This can be a pathogen (virus, bacteria), a specific protein, or a synthetic peptide.

2. Extraction:

Depending on the source, different methods are used to extract the antigen: **Bacteria or Viruses, Tissues, Synthetic Peptides.**

3. Purification:

- **Centrifugation:** Separates components based on size and density.
- **Filtration:** Uses filters to remove contaminants.
- **Chromatography:** Techniques like affinity, ion exchange, and size-exclusion chromatography are used to purify proteins.
- **Electrophoresis:** Separates proteins based on size and charge.

4. Characterization:

- **SDS-PAGE:** To check the molecular weight and purity.
- **Mass Spectrometry:** For precise molecular weight determination.
- **Western Blotting:** To verify the presence and specificity of the antigen.
- **ELISA:** To check the reactivity of the antigen with specific antibodies.

5. Storage:

Proper storage is crucial to maintain antigen stability and activity:

- **Lyophilization (Freeze-drying):** To extend shelf-life.
- **Cryopreservation:** Storing at ultra-low temperatures.
- **Buffer Solutions:** Using appropriate buffers to maintain pH and prevent degradation.

Applications of Prepared Antigens

- **Vaccine Development:** Prepared antigens are used to stimulate the immune system and develop immunity against specific pathogens.
- **Diagnostic Tests:** Used in assays like ELISA to detect the presence of antibodies or pathogens.
- **Research:** Study immune responses and disease mechanisms.

PREPARATION OF ANITSERA

Steps in Raising Antibodies

1. Antigen Preparation: Before starting the immunization process, the antigen must be prepared. It should be pure and properly characterized.

Purification: Ensure the antigen is free from contaminants using techniques like chromatography.

Characterization: Verify the antigen's identity and purity using methods such as SDS-PAGE, mass spectrometry, or Western blotting.

2. Adjuvant Selection: Adjuvants are substances that enhance the immune response to the antigen. Commonly used adjuvants include:

- **Freund’s Adjuvant**: Complete (CFA) for the initial injection and Incomplete (IFA) for booster injections.
- **Alum**: Aluminum hydroxide or phosphate, commonly used in vaccines.
- **Liposomes and Microparticles**: Enhance the delivery and presentation of the antigen.

3. Immunization Protocol

- **Animal Selection**: Commonly used animals include mice, rabbits, goats, and sheep. The choice depends on the required antibody volume and application.
- **Initial Immunization**: Inject the antigen-adjuvant mixture subcutaneously or intramuscularly to initiate the immune response.
- **Booster Immunizations**: Administer booster doses at regular intervals (typically 2-4 weeks) to enhance and sustain the immune response.
- **Monitoring Response**: Collect blood samples at intervals to monitor antibody production using assays like ELISA.

4. Antibody Collection and Purification

- **Serum Collection**: For polyclonal antibodies, collect blood and separate the serum by centrifugation.
- **Hybridoma Production**: For monoclonal antibodies, fuse the B cells from immunized mice with myeloma cells to create hybridomas. Screen and select hybridomas producing the desired antibody.

5. Antibody Purification: Purify the collected antibodies to remove non-specific proteins and other contaminants.

- **Affinity Chromatography**: Use antigen-specific columns to purify antibodies.
- **Ion Exchange and Size-Exclusion Chromatography**: Additional purification steps to enhance antibody purity.

6. Characterization and Validation: Characterize and validate the purified antibodies to ensure their specificity and functionality.

- **ELISA:** To determine antibody titer and specificity.
- **Western Blotting:** To confirm the antibody can recognize the antigen in a complex mixture.
- **Flow Cytometry:** To test the antibody's ability to bind to cell surface antigens.
- **Immunohistochemistry:** To assess the antibody's performance in tissue samples.

ROUTES OF ADMINISTRATION

Routes of Administration for Antigens

1. Intramuscular (IM)

- Description: Injection into the muscle tissue.
- Advantages: Good for vaccines; allows slow and sustained release of antigen.
- Common Sites: Deltoid muscle, thigh muscle.
- Applications: Many vaccines such as the influenza vaccine.

2. Subcutaneous (SC)

- Description: Injection into the layer of fat just beneath the skin.
- Advantages: Easier to administer than IM; good for slow absorption.
- Common Sites: Upper arm, thigh, abdomen.
- Applications: Insulin administration, some vaccines.

3. Intravenous (IV)

- Description: Injection directly into the bloodstream.
- Advantages: Immediate systemic distribution; useful for rapid response.
- Common Sites: Veins in the arm.
- Applications: Emergency treatments, some cancer therapies.

4. Intradermal (ID)

-Description: Injection into the dermis, just below the epidermis.

-Advantages: requires a smaller dose.

-Common Sites: Forearm.

-Applications: Tuberculosis skin test (Mantoux test), some vaccines.

5. Oral

-Description: Administration through the mouth.

-Advantages: Non-invasive; easy to administer.

-Challenges: Antigens may be degraded by stomach acids.

-Applications: Oral vaccines like the polio vaccine.

6. Nasal

- -Description: Administration through the nasal passages.
- -Advantages: Non-invasive; targets mucosal immunity.
- -Applications: Nasal spray vaccines like the flu vaccine.

7. Transdermal

- -Description: Administration through the skin using patches.
- -Advantages: Non-invasive; provides a controlled release.
- -Applications: Nicotine patches, some experimental vaccines.
- (Methimazole-to treat hyperthyroidism)
- (Amitriptyline- behavioural issues and pain relief)
- (Fentanyl- pain relief)

Routes of Administration for Antibodies

1. Intravenous (IV)

- - Description: Injection directly into the bloodstream.
- - Advantages: Immediate systemic distribution; high bioavailability.
- - Common Sites: Veins in the arm.
- - Applications: Therapeutic monoclonal antibodies for cancer, autoimmune diseases.

- **2. Subcutaneous (SC)**
- -Description: Injection into the layer of fat just beneath the skin.
- -Advantages: Easier self-administration; slow and sustained release.
- -Common Sites: Upper arm, thigh, abdomen.
- -Applications: Monoclonal antibodies for chronic conditions like rheumatoid arthritis.

3. Intramuscular (IM)

-Description: Injection into the muscle tissue.
-Advantages: Allows slower release than IV; good for depot preparations.
-Common Sites: Deltoid muscle, thigh muscle.
-Applications: Some therapeutic antibodies and vaccines.

4. Intrathecal

-Description: Injection into the spinal canal.
-Advantages: Direct delivery to the central nervous system.
-Common Sites: Spinal canal.
-Applications: Treatment of certain central nervous system infections, some experimental therapies.

5. Intraperitoneal (IP)

- Description: Injection into the peritoneal cavity.
- Advantages: Good for animal studies; can be used for localized treatments.
- Applications: Research applications, some experimental cancer treatments.

6. Topical

-Description: Application to the skin or mucous membranes.
-Advantages: Localized effect; non-invasive.
-Applications: Antibody creams for wound healing, eye drops for ocular conditions.

Doses for Administration: Antigens and Antibodies

- The dose of an antigen or antibody administered can vary significantly depending on the purpose (e.g., vaccination, therapeutic treatment), the specific substance, the route of administration, and the recipient's characteristics.
- Below, we'll discuss typical dosing considerations for both antigens and antibodies.

Doses for Antigen Administration

1.Vaccination

1. **Intramuscular (IM)**: **Example**: Influenza vaccine- **Dose**: 0.5 mL (adult dose); 0.25 mL (children under 3 years).
2. **Subcutaneous (SC)**: **Example**: Measles, Mumps, Rubella (MMR) vaccine- **Dose**: 0.5 mL.
3. **Intradermal (ID)**: **Example**: Tuberculosis (BCG) vaccine- **Dose**: 0.1 mL.
4. **Oral**: **Example**: Oral Polio Vaccine (OPV)- **Dose**: 2-3 drops (about 0.1 mL).
5. **Nasal**: **Example**: Nasal influenza vaccine- **Dose**: 0.2 mL (0.1 mL per nostril).

2. Research and Experimental Uses: **Animal Studies**: Doses can vary widely based on the experimental design.

- **Example**: Protein antigens in mice.
- **Dose**: Typically 10-100 µg per injection, depending on the protocol.

Doses for Antibody Administration

1.Therapeutic Use

1. Intravenous (IV)

1. **Example**: Monoclonal antibody therapies for cancer (e.g., Rituximab).
2. **Dose**: 375 mg/m² weekly for 4 weeks.

2. Subcutaneous (SC)

1. **Example**: Monoclonal antibody for chronic conditions (e.g., Adalimumab for rheumatoid arthritis).
2. **Dose**: 40 mg every other week.

3. Intramuscular (IM)

1. **Example**: Immunoglobulin replacement therapy.
2. **Dose**: 1-2 grams every 3-4 weeks.

2. Prophylactic Use

- **Intravenous (IV)**
  - **Example**: Immune globulin for exposure to infectious diseases.
  - **Dose**: 0.1-0.4 g/kg body weight.
- **Intramuscular (IM)**
  - **Example**: Rabies immune globulin.
  - **Dose**: 20 IU/kg body weight.

3. Diagnostic Use

- **Intravenous (IV)**
  - **Example**: Radiolabeled antibodies for imaging.
  - **Dose**: Varies depending on the specific diagnostic agent and protocol.

Factors Influencing Dosage

1.Purpose of Administration

1. **Preventive (Vaccination)**: Typically standardized doses to elicit an immune response.
2. **Therapeutic (Treatment)**: Doses tailored to achieve therapeutic levels and manage the disease.
3. **Diagnostic**: Doses optimized for imaging or detection without adverse effects.

2.Recipient Characteristics

1. **Age and Weight**: Children often require lower doses; dose adjustments for body weight.
2. **Health Status**: Immunocompromised individuals might need different dosing.
3. **Previous Exposure**: History of vaccination or antibody exposure can influence dosing needs.

Purification of Antibodies: Methodology for IgG and IgA

Purification of IgG

1. Source Material Preparation

•Serum/Plasma: Typically used for polyclonal IgG.
•Cell Culture Supernatant: Used for monoclonal IgG production from hybridoma cells.
•Tissue Lysate: For antibodies extracted from tissues.

2. Initial Clarification

•Centrifugation: Spin the sample at 10,000-15,000 x g for 15-30 minutes to remove cells and debris.
•Filtration: Use a 0.45 µm or 0.22 µm filter to further clarify the supernatant.

3. Protein A/G/L Affinity Chromatography

•**Principle**: Protein A/G/L binds to the Fc region of IgG with high affinity, allowing for specific capture of IgG.
•**Procedure**:

- **Column Preparation**: Equilibrate the Protein A/G/L column with binding buffer (e.g., PBS, pH 7.4).
- **Sample Loading**: Apply the clarified sample to the column.
- **Washing**: Wash the column with binding buffer to remove non-specifically bound proteins.
- **Elution**: Elute IgG with a low pH buffer (e.g., glycine-HCl, pH 2.7-3.0). Immediately neutralize the eluate with a neutralizing buffer (e.g., Tris-HCl, pH 9.0).

4. Desalting and Buffer Exchange

•**Dialysis**: Dialyze the eluted IgG against a suitable buffer (e.g., PBS) to remove salts and other small molecules.

•**Size-Exclusion Chromatography**: Alternatively, use a size-exclusion column to exchange buffers and remove aggregates.

5. Purity Assessment

•**SDS-PAGE**: Analyze the purity of the IgG by SDS-PAGE.

•**Western Blotting**: Confirm the identity and purity using a Western blot with an anti-IgG antibody.

•**ELISA**: Quantify the IgG concentration and check for specificity.

Purification of IgA

1. Source Material Preparation

- **Colostrum/Saliva**: Common sources of IgA.
- **Serum/Plasma**: Less common due to lower concentrations of IgA compared to IgG.
- **Cell Culture Supernatant**: For monoclonal IgA production.

2. Initial Clarification

- **Centrifugation**: Spin the sample at 10,000-15,000 x g for 15-30 minutes to remove cells and debris.
- **Filtration**: Use a 0.45 µm or 0.22 µm filter to further clarify the supernatant.

3. IgA-Specific Affinity Chromatography

•**Principle**: Capture IgA using ligands that specifically bind to the Fc or Fab regions of IgA.
•**Procedure**:

- **Column Preparation**: Equilibrate the IgA affinity column (e.g., jacalin-agarose) with binding buffer (e.g., PBS, pH 7.4).
- **Sample Loading**: Apply the clarified sample to the column.
- **Washing**: Wash the column with binding buffer to remove non-specifically bound proteins.
- **Elution**: Elute IgA with a specific elution buffer (e.g., galactose or a low pH buffer). Neutralize the eluate if using a low pH buffer.

4. Alternative Methods for IgA Purification

•**Immobilized Metal Affinity Chromatography (IMAC)**: Utilize metal-chelating ligands to purify IgA tagged with polyhistidine.
•**Ion Exchange Chromatography**: Separate based on charge differences; useful as a polishing step after affinity purification.

5. Desalting and Buffer Exchange

•**Dialysis**: Dialyze the eluted IgA against a suitable buffer (e.g., PBS) to remove salts and other small molecules.

•**Size-Exclusion Chromatography**: Alternatively, use a size-exclusion column to exchange buffers and remove aggregates.

6. Purity Assessment

•**SDS-PAGE**: Analyze the purity of the IgA by SDS-PAGE.

•**Western Blotting**: Confirm the identity and purity using a Western blot with an anti-IgA antibody.

•**ELISA**: Quantify the IgA concentration and check for specificity.

Monoclonal Antibody Raising -Hybridoma Technology

- Monoclonal antibodies (mAbs) are highly specific antibodies produced by identical immune cells that are clones of a unique parent cell.
- Hybridoma technology is the most common method used to produce monoclonal antibodies.
- This technology involves the fusion of an antibody-producing B cell with a myeloma (cancer) cell to create a hybridoma, which can be cultured indefinitely to produce large quantities of the monoclonal antibody.

Steps in Raising Monoclonal Antibodies Using Hybridoma Technology

1. Immunization

•**Antigen Preparation**: The antigen should be pure and well-characterized. It can be a protein, peptide, or other molecules.

•**Adjuvant**: Often, an adjuvant such as Freund's complete adjuvant (CFA) for the initial immunization and Freund's incomplete adjuvant (IFA) for subsequent boosts are used to enhance the immune response.

•**Immunization Schedule**: Mice are typically used for immunization. The schedule involves:

- Initial injection with the antigen-adjuvant mixture.
- Booster injections at regular intervals (e.g., every 2-3 weeks).
- Final boost without adjuvant before harvesting spleen cells.

2. Cell Fusion

•**Harvesting Spleen Cells**: After the final boost, the mouse is sacrificed, and spleen cells, which include B cells producing the desired antibody, are collected.

•**Myeloma Cells**: Use myeloma cells (e.g., SP2/0 or NS0) that are immortal.

•**Fusion Process**:

- Mix spleen cells with myeloma cells in the presence of a fusogen, such as polyethylene glycol (PEG).
- Incubate to allow cell fusion.

3. Selection of Hybridomas

•**HAT Medium**:
•The fused cells are cultured in hypoxanthine-aminopterin-thymidine (HAT) medium.
•Unfused myeloma cells die because they lack the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT), and unfused B cells die because they are not immortal.
•**Screening**:
•After a few weeks, hybridomas are screened for the production of the desired antibody using assays such as ELISA or immunofluorescence.

4. Cloning

•**Limiting Dilution**: Positive hybridomas are diluted and plated to isolate single cells, ensuring that each colony arises from a single hybridoma cell.
•**Expansion**: Clones producing the desired antibody are expanded in culture.

5. Antibody Production

•**In Vitro**: Hybridomas are cultured in bioreactors or large culture flasks to produce antibodies.
•**In Vivo**: Alternatively, hybridomas can be injected into the peritoneal cavity of mice to produce ascites fluid rich in monoclonal antibodies.

6. Purification of Monoclonal Antibodies

•**Affinity Chromatography**: Use Protein A/G/L columns to purify IgG from the culture supernatant or ascites fluid.

•**Desalting and Buffer Exchange**: Dialyze the purified antibodies against the desired buffer.

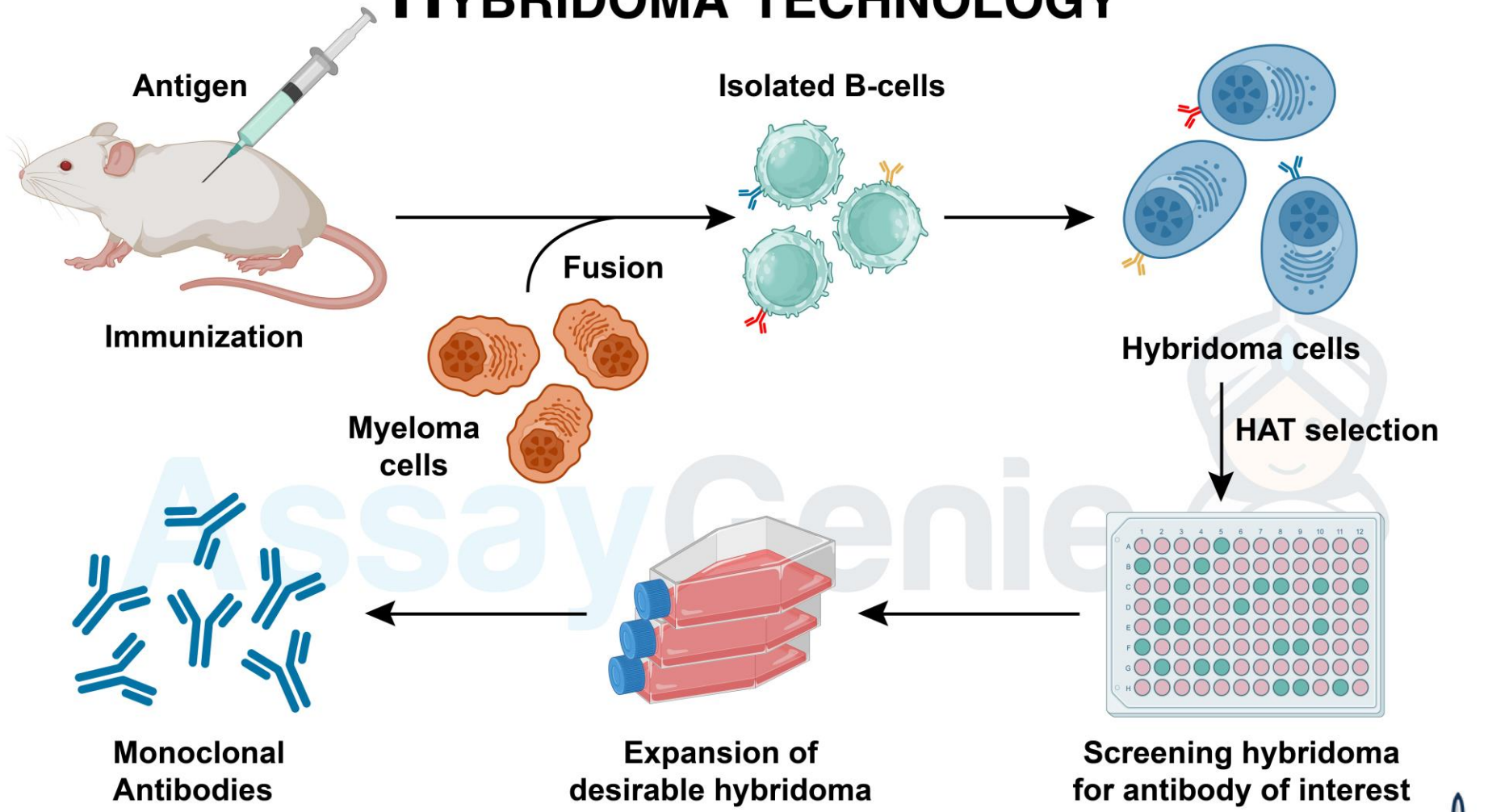
7. Characterization and Validation

•**SDS-PAGE and Western Blotting**: Assess purity and verify the size of the antibody.

•**ELISA**: Determine antibody concentration and specificity.

•**Functional Assays**: Confirm the antibody's ability to bind to the antigen in various applications (e.g., flow cytometry, immunohistochemistry).

HYBRIDOMA TECHNOLOGY



Applications of Monoclonal Antibodies

1.Research: Used in various immunoassays, Western blotting, immunofluorescence, and immunoprecipitation.

2.Diagnostics: Essential for developing diagnostic tests such as ELISA, lateral flow assays, and immunohistochemistry.

3.Therapeutics: Monoclonal antibodies are used as treatments for cancers, autoimmune diseases, and infectious diseases.

Advantages

•**Specificity**: Produces highly specific antibodies against a single epitope.

•**Consistency**: Hybridomas can be cultured indefinitely, providing a consistent source of antibodies.

•**Versatility**: Can be used to produce antibodies against a wide range of antigens.

ACKNOWLEDGEMENT

- The presentation is being used for educational and non-commercial purposes.
- Thanks are due to all the original contributors and entities whose pictures were used to create this presentation.