



IMMUNOCHEMICAL TECHNIQUES

24.1 INTRODUCTION

All vertebrates have advanced immune system. The more complex the organism the more advanced the immune system. The immune system of mammals has evolved over a million years; it provides an incredible protection system capable of responding to infective challenges that arise in the body. Immunity in our body is monitored or controlled by specific cells from stem cells in bone marrow. The most important cell types are B and T lymphocytes, which have the ability to act against bacterial and other viruses. B-cells releases **antibody** against stimulation of a foreign substance into the body. An **antigen** is a foreign substance capable of an immune response leading to the production of antibodies. They are the targets to which antibodies bind. So antibodies are antigen specific (bind to the antigen that initiated its production). This unit focuses on the identification and diagnostic chemical techniques on the response of an antibody to a specific antigen. **Immunochemical methods** are based on the selective, reversible and non-covalent binding of antigens by antibodies. These methods are employed to detect or quantify either antigens or antibodies.



OBJECTIVES

After reading this lesson, you will be able to:

- define immunochemical techniques
- explain the characteristic features and roles of antigen – antibody reactions
- describe and distinguish the types of immunochemical techniques

24.2 FACTORS CONTROLLING THE IMMUNOCHEMICAL TECHNIQUES

All the techniques cannot be used for the identification of a specific antigen or antibody. In order to perform the immunochemical techniques, several controlling criteria are appropriate:

- Experimental conditions – nature and the place of work, type of sample collected.
- Nature of Reagents – the quality is studied, standardized and analyzed
- Sensitivity and selectivity of technique to the particular sample

24.3 IMMUNOCHEMICAL TECHNIQUES

Immunochemistry is an advanced area of immunology. It deals with the chemical components and chemistry (chemical reactions) of immunological phenomena, that is of antibody and antigen. Immunochemical methods are processes utilizing the highly specific affinity of an antibody for its antigen. It detects the distribution of a given protein or antigen in tissues or cells. The methods used for the immunochemical analysis are called Immunochemical techniques; they are highly important in diagnostic and clinical context, as now even normal cell with many proteins are altered in diseased state (in cancer).

24.3.1 Characteristics and Role

24.3.1.1 Characteristics

- Simple, rapid and robust
- Highly sensitive
- Easily automated – applicable to regular clinical diagnostic laboratories
- Does not require extensive and easily destructible sample preparation
- Do not require expensive instrumentation
- Mostly based on simple photo-, fluoro- and luminometric detection
- Measurements may be either qualitative or quantitative.

24.3.1.2 Roles

The characteristic features of these techniques are helpful for the following:

- Function of newly identified or novel proteins can be identified
- Importance of uncharacterized protein in their natural environment can be analyzed



Notes

MODULE

Biochemistry



Notes

Immunochemical Techniques

- Determination of species or tissues in which the protein or residues is expressed
- The cell type or sub-cellular compartment in which the protein can be found
- Detect whether there is any variation in expression of protein during development of the organism
- Some alteration in the normal expression pattern of a particular protein may indicate a particular disease state; Gives information that may be useful for diagnosis and treatment.

24.4 METHODS OF ANALYSIS

All immunochemical methods are based on a highly specific and sensitive reaction between an antigen and an antibody. Antibodies are immunoglobins, belonging to a family of glycoproteins – IgG, IgA, IgD, IgM and IgE. Structurally, antibodies are often visualized as Y-shaped molecules, each containing 4 polypeptides – 2 identical polypeptide units called heavy chains and another 2 called light chains. It has a domain called Fab, the site where it binds to an antigen. The region of an antigen binding to an antibody is called an epitope (Fig. 24.1).

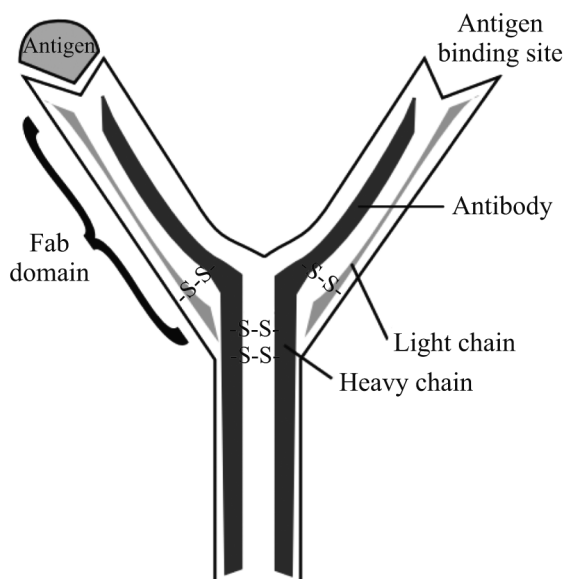


Fig. 24.1: Antigen-Antibody reaction

The measure of the strength of the binding is called **affinity**, and it is usually expressed in terms of the concentration of an antibody-antigen complex measured at equilibrium. It is measured by **quantitative precipitin curve** (basis for many immunochemical techniques) proposed by Heidelberger and Kendall in 1935.

Quantitative precipitin curve: it describes the relationship between the antigen concentration and the amount of precipitate for a constant quantity of an antibody. Three zones can be distinguished from the precipitin curve: (Fig. 24.2)



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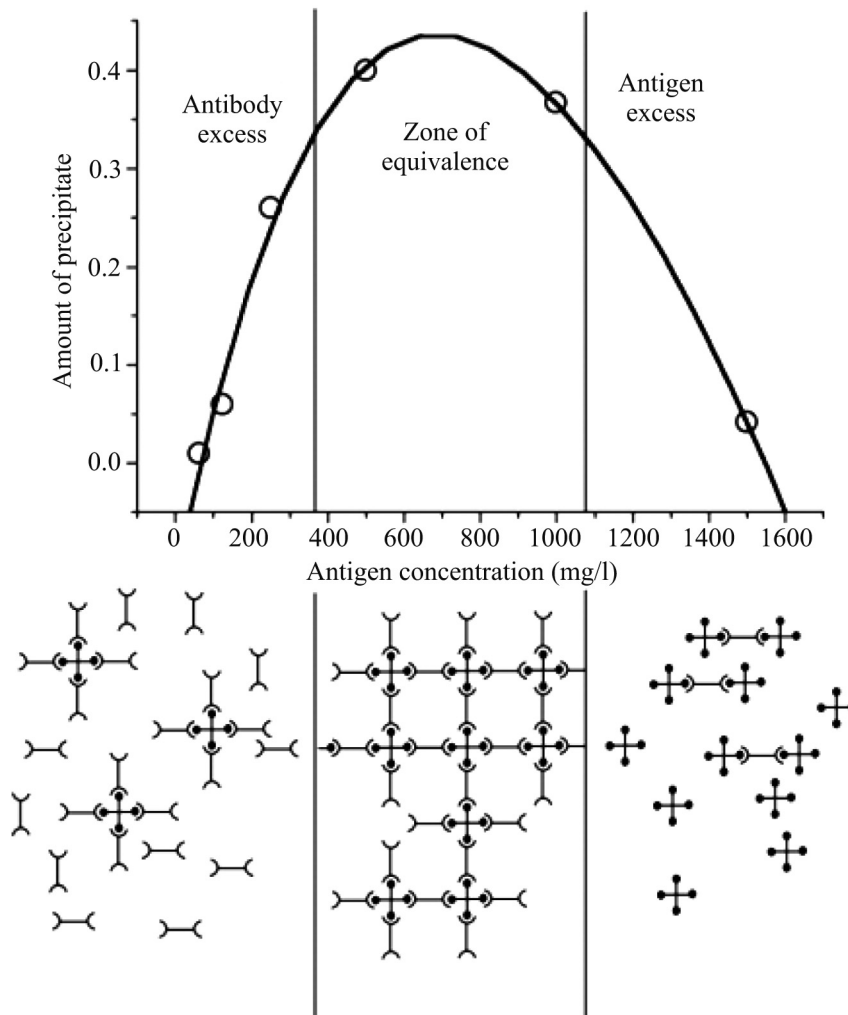


Fig. 24.2: Precipitin curve

- (i) antibody excess zone – first phase where less antigen is present in sample
- (ii) equivalence zone – both antigen and antibody are cross-linked forming precipitate; no free antigen or antigen is present
- (iii) antigen excess zone – amount of precipitate reduces due to high antigen concentration

The precipitin curve forms the basis of most of the immunochemical techniques that can be performed in clinical laboratories.



Notes



INTEXT QUESTIONS 24.1

1. cells releases antibody against stimulation of a foreign substance into the body
2. is a foreign substance capable of inducing the production of antibodies
3. methods detect or quantify either antigen or antibodies
4. The measure of the strength of the binding is called

24.4.1 Types of Antibody Used

The antibodies used for the methods are produced by various ways:

1. **Monoclonal antibody** – products of single clone of plasma cells by B-lymphocytes; mostly prepared in laboratory. They are directed against single epitope – identical copies with same structure and antigen specificity. They have excellent specificity but poor ability to precipitate antigen.
2. **Polyclonal antibody** – they are conventional, i.e., produced by immunization of animals with antigen. Thus antibody consists of mixture of monoclonal antibodies having specificity for complex antigens.

Sometimes monoclonal is called as “monovalent” and polyclonal as “polyvalent” which indicates the antigen specificity.

24.4.2 Types

Based on the type of reaction performed, reagents and samples used, the techniques are categorized as follows:

- Particle methods – where the antigen-antibody interaction is observed. It includes:
 - Agglutination
 - Immunoprecipitation
 - Immunoelectrophoresis
 - Immunofixation
 - Immunoturbidimetry
 - Immunonephlometry
- Label methods – either antigen or antibody is labeled and through the label concentration, the antigen-antibody reaction is observed. This includes:

- Immunoassay
- Competitive binding
- Other methods – includes immunofluorescence, immunoelectron microscopy, etc.

24.4.2.1 Agglutination

Agglutination (from Latin, *agglutino* – to glue/ attach) is a process of formation of clumping of cells; it occurs due to reaction of antibody on a particulate antigen (Fig. 24.3). Among all other antibodies, IgM is a good agglutinin, since it has high affinity to different antigens.

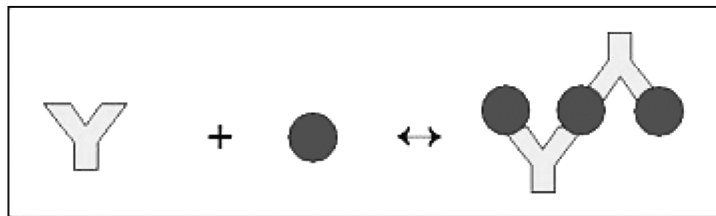


Fig. 24.3: Agglutination reaction

They may be either:

- Qualitative test – to identify the presence of an antigen or an antibody.
- Quantitative test – to measure the level of antibodies to particulate antigens; serial dilutions of sample is used.

There are many variations in agglutination method:

- Direct – antigen present on cell surface is directly agglutinated by specific antibodies.
Eg: in hematology – determination of blood group (ABO typing) diagnosis of *Salmonella typhi* – *Widal Test*
- Indirect (reverse agglutination) – uses particles already coated with antigens (antibodies) to determine the antibody (antigen) in given sample. Eg: Pregnancy test – using hormone secretion (human chorionic gonadotropin), diagnosis of rheumatoid arthritis
- Agglutination inhibition – where competitive binding of antigen occurs; determine the concentration of soluble antigen in sample.
- Haemagglutination – involve reactions using red blood cells; detection of diseases and other viruses, blood typing, etc.





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INTEXT QUESTIONS 24.2

1. Types of antibody used are &
2. is a process of formation of clumping cells
3. Example of Direct agglutination is typing
4. Example of Indirect agglutination is test

24.4.2.2 Immunoprecipitation

Immunoprecipitation methods include flocculation and precipitation reactions. When a solution of an antigen is mixed with its corresponding antibody under suitable conditions, the reactants form flocculating or precipitating aggregates. They can be assessed visually by the formation of precipitin line at the region of equivalence – where equivalent amount of antigen and antibody are present.

It may be either:

- Simple – reaction of one antigen and an Precipitin line antibody
- Complex – when many unrelated reactants are used

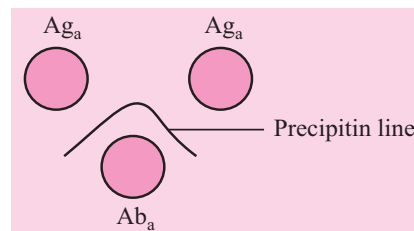


Fig. 24.4: Precipitation reaction

In *simple methods*, a concentration gradient is established between the antigen and antibody; it includes:

- Single radial immune diffusion (SRID) – developed by Mancini; simple technique where the circular precipitin area originating from antigen well in gel is equal to amount of antigen concentration.
- Double immune diffusion – developed by Orjan Ouchterlony; both antigen and antibody diffuse from separate wells in a gel to form precipitin line.

In *complex methods*, different antigens are compared with an antibody and vice versa. The result is based on the presence or absence of precipitin line.

24.4.2.3 Immunelectrophoresis

Immunelectrophoresis is a qualitative technique that combines of two methods one after another:

- Gel electrophoresis – separation of components by charge (Fig. 24.5a)
- Immunodiffusion – diffusion of both antibody and antigen toward each other produces precipitin line (Fig. 24.5b)

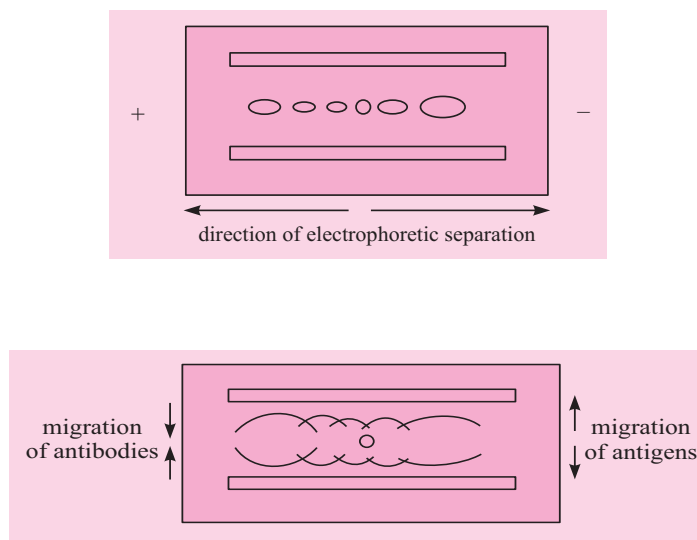


Fig. 24.5: (a) electrophoresis, (b) immunodiffusion

It is strictly *qualitatively* technique to detect antibody and antigen in sample. But also a *quantitative* technique exist called **rocket immunelectrophoresis** to measure the antigen level in a sample.

Another variation called as counter-current immunelectrophoresis where it is similar to double immunodiffusion.

24.4.2.4 Immunofixation

Immunofixation is used for detection and identifying antibody in serum, urine and other body fluids (Fig. 24.6). The principle is similar to immunelectrophoresis, involves two stages:

- Electrophoresis separation of antibody proteins
- Immunoprecipitation separation with specific antibodies.

It is easy to perform, more sensitive and easily evaluated. In clinical laboratories, is used for detection for cancerous myeloma through specific antibody.



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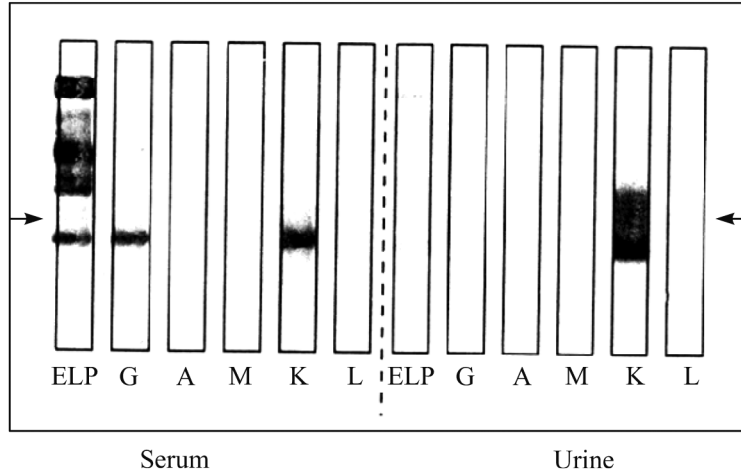


Fig. 24.6: Immunofixation – serum and urine samples

24.4.2.5 Immunturbidimetry

The precipitin line or curve that forms in a gel, can also be formed in a solution. When an antigen solution is combined with a specific antibody solution, the clumping formed results in a cloudy appearance. This is called turbidity; it forms the principle of immunturbidimetry. It is measured based on the intensity/ amount of light that pass through the sample. It is performed by an instrument called spectrophotometer (Fig. 24.7-A).

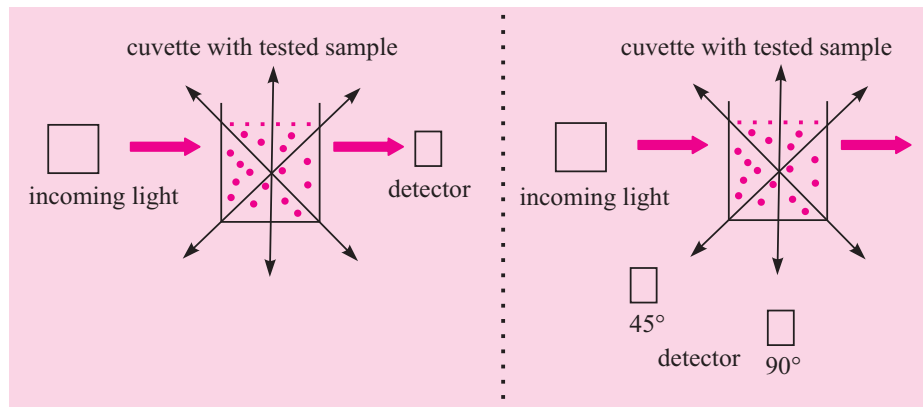


Fig. 24.7: A – turbidimetry, B – nephelometry

24.4.2.5 Immunonephelometry

Immunonephelometry works on the principle similar to turbidimetry, but this method measures the intensity of scattered light from sample directly. It uses laser light and is measured by using a nephelometer (Fig. 24.7-B).

Both the methods are faster but are more expensive. Increased sensitivity of antigen and antibody can be got by automation of these techniques.

24.4.2.7 Immunoassay

Immunoassay technique works on the principle of labeling either the antigen or antibody before the reaction. This increases the sensitivity of detection in the experiment than the formation of immunoprecipitate.

The label can be of:

- Radio isotope – called as Radio Immunoassay (RIA)
- Enzyme – reaction known as Enzyme Immunoassay (EIA); it also includes ELISA (Enzyme Linked Immuno Sorbent Assay)
- Fluorescent substance
- Chemiluminescent substance

RIA – first immunoassay technique; based on the measurement of radioactivity associated with antigen-antibody complexes.

ELISA – safer and efficient method; based on the measurement of an enzyme action associated with antigen-antibody complexes (Fig. 24.8) Most commonly used enzymes – biotin, alkaline phosphatase, etc.

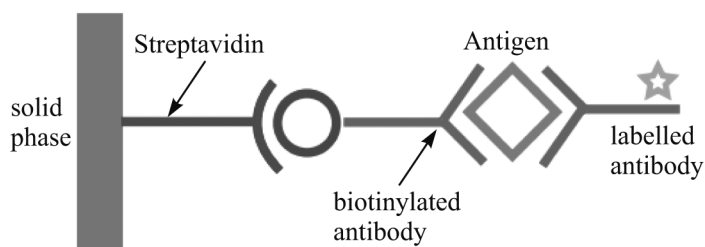


Fig. 24.8: ELISA – sandwich type

24.4.2.8 Competitive Binding

Another method to measure the amounts of antigen is competitive binding; in this technique, it works on the principle of competitive binding:

- Antibody is first incubated in solution having antigen
- This mixture is added to antigen coated reaction well

The more the antigen present in sample, the less the antibody will bind to the coated antigen. To this, when labeled antibody is added, we can measure the amount of antigen present in sample.



Notes



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24.4.2.9 Other Methods

- **Immunofluorescence** – an antibody labeled with a fluorescent molecule (fluorescein or rhodamine or one of many other fluorescent dyes) is used to detect the presence of an antigen in or on a cell or tissue by the fluorescence emitted by the bound antibody.
- **Immunoelectron microscopy** – uses the specificity of antibody to view tissues and its components through a microscope.
- **Immunostaining** – use of antibody labeled with enzyme or dye to detect specific protein in a sample. Most important staining method is **Western Blot** method that includes three processes:
 - Electrophoretic separation of proteins in sample
 - Immunoblotting – transfer of proteins from gel to a membrane
 - Immunodetection – identification using labeled antibody

24.5 DETECTION

In order to detect and diagnose diseases and also certain body conditions properly, there are many fast immunochemical techniques. Some examples are:

- Detection of *Salmonella typhi* (causing typhoid) in blood
- Detection of narcotics in urine and saliva
- Detection of protein hormones like insulin, thyroxine, etc.
- Pregnancy test for level of human chorionic gonadotropin hormone (hCGH)



WHAT HAVE YOU LEARNT

- Immunity in our body is monitored or controlled by specific cells from stem cells in bone marrow.
- The most important cell types are B and T lymphocytes, which have the ability to act against bacterial and other viruses.
- B-cells release antibody against stimulation of a foreign substance into the body.
- An antigen is a foreign substance capable of an immune response leading to the production of antibodies
- Immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies

- The methods used for the immunochemical analysis are called Immunochemical techniques
- All immunochemical methods are based on a highly specific and sensitive reaction between an antigen and an antibody
- Antibodies are immunoglobins, belonging to a family of glycoproteins – IgG, IgA, IgD, IgM and IgE
- The measure of the strength of the binding is called affinity
- Quantitative precipitin curve describes the relationship between the antigen concentration and the amount of precipitate for a constant quantity of an antibody
- Types of antibodies used are Monoclonal antibody and Polyclonal antibody
- Based on the type of reaction performed, reagents and samples used, the techniques are categorized as follows:
- Particle methods – where the antigen-antibody interaction is observed. It includes:
 - Agglutination
 - Immunoprecipitation
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- Label methods – either antigen or antibody is labeled and through the label concentration, the antigen-antibody reaction is observed. This includes:
 - Immunoassay
 - Competitive binding
- Other methods – includes immunofluorescence, immunoelectron microscopy, etc.



TERMINAL QUESTIONS

1. Give an account of characteristics of immunochemical techniques.
2. What are the different methods of analysis?



MODULE

Biochemistry

Immunochemical Techniques



Notes

3. Discuss the various methods of agglutination.
4. Enumerate the differences between immunoturbidimetry and immunonephlometry.
5. Compare immunoprecipitation and immunoelectrophoresis.
6. Give the importance of immunoassay. Mention its types.



ANSWERS TO INTEXT QUESTIONS

24.1

1. B
2. Antigen
3. Immunochemical
4. Affinity

24.2

1. Monoclonal & Polyclona
2. Agglutination
3. ABO
4. Pregnancy