LECTURE NOTES

For Medical Laboratory Technology Students

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Immunology and Serology

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Preface

Immunology and serology is an advanced science dealing with how the human immune system organized, function and the different types of serological techniques. It is a very vast subject covering a wide area of technology.

The shortage of reference materials in the area and in order to present the subject in a relatively simplified and organized way called the need for preparing a lecture note.

This teaching material is prepared based on the existing curriculum of immunology and serology and consists of 16 chapters. Each chapter has its own objective, body and questions (exercises) at the end. Therefore, the material is designed to present clear and concise understanding about immunology and serology; and it is primarily suitable for students following diploma programme in medical laboratory technology.

Finally, it is quite obvious that it had demanded a lot of effort in preparing this material. However, it should be noted that even then, there could be constructive comments which are helpful in improving this lecture note.Thus, it will be well accepted and acknowledged for the contribution.

Acknowledgments

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Finally, I wish to extend my thanks to W/t Aschalech Temesgen for writing me the draft of this lecture note.

Abbreviations

CRP	-	C-reactive Protein
EBV	-	Epstien-Barr Virus
EIA	-	Enzyme Immune Assay
HCG	-	Human Chorionic Gonadotrophin
PMN	-	Polymorphonuclear Leukocytes

Table of Contents

CHAPTER ONE

INTRODUCTION TO IMMUNOLOGY-SEROLOGY

At the end of this chapter, the reader should be able to:

- Define the term immunology
- Describe the historical background of immunology

1.1 Historical Background of Immunology

Immunology is defined as the study of the molecules, cells, organs, and systems responsible for the recognition and disposal of foreign material. Immunology began as a branch of microbiology. The study of infectious disease and the body's response to them has a major role for the development of immunology. More over, the concept of germ theory of disease has contributed to the field of immunology.

It was Edward Jenner who first studied the response of the body to foreign substances. He observed that dairy maids who had naturally contracted a mild infection called cowpox seemed to be protected against smallpox, a horribly disfiguring disease and a major killer.

In 1796, Jenner inoculated an eight year-old boy with fluid from cowpox blisters on the hand of a dairymaid. The boy contracted cowpox. Then two month later Jenner inoculated him with fluid from a small pox blister, the boy only developed a small sore at the site of inoculation. His exposure to the mild disease cowpox had made him immune to the small pox infection. These were some of the vital events occurred in the history of immunology following Jenner's achievement.

In 1879, the first human pathogen, gonococcus, was isolated by Neisser. In 1883, Klebs and Loeffler isolated diphtheria bacilli which led to the production of the first defined antigen, diphtheria toxin, by Roux and Yersin in 1888. In the same year the first antibodies, serum bactericidins, were reported by Nuttal and Pasteur.

In 1890, von Behring and Kitasato discovered antitoxins that led to the development of toxoids for diphtheria and tetanus. In 1900, Land Steiner discovered the blood group antigens and their corresponding antibodies. This led to the ability to give blood transfusion with out provoking reactions. It was in 1916 that the first journal of immunology began publication in which many of new findings published on it. In general, immunology has always depended on and stimulated the application of technology, such as the use of microscopy, electrophoresis, immunoelectrofluorescence, etc. Thus

immunology has not become an inborn discipline but has maintained close associations with many other fields of medical sciences.

Review Questions

1. Who was the first person studied the body's response to foreign substance?
2. Describe the development of the field immunology
3. What was the contribution of Land Steiner for the field of immunology?

CHAPTER TWO

IMMUNITY

Learning Objectives

At the end of this chapter, students are expected to:

- Describe the different types of immunity
- Explain the role of the immunity in defense mechanism
- Discuss factor that affect the immunity

2.1. Definition

Immunity can be defined as the way in which the body can protect itself from invasion by pathogenic microorganism and provide a defense against their harmful effect.

Immunity is classified in to two major groups

- Non specific immunity
- Specific immunity

2.1.1. Non specific (natural or innate) immunity.

Non-specific immunity, also called natural or innate immunity, is the first line of defense against any infectious agent. Non specific host responses provide an effective barrier that

prevents the microorganisms from penetrating, inhibit or destroy the invader if it gains access to the tissues, and eliminate or neutralize any toxic substance elaborated by infectious agent. Several mechanisms are available in the immunocompetent host. These include physical or mechanical barrier, biochemical factors, cellular mechanism, role of normal flora & inflammatory reactions.

Physical or mechanical barrier

The unbroken skin and mucus membrane are effective mechanical barriers to infectious agents. The surface of the skin is also inhibitory to the growth of most microorganisms because of low moisture, low pH, and the presence of secreted inhibitory substance. However, it is possible for some microorganisms to enter the skin through hair follicles, sebaceous glands or sweet glands.

Similarly, mucus membranes consist of an epithelial layer and an underlying connective tissue layer. They line the entire digestive, respiratory, urinary, and reproductive tracts. For example, the epithelial surface that lines the nasal cavity and throat are protected by a combination of mucous production and cilliary movement. Because mucous is so viscous, microorganisms adhere to it. Epithelial cells with cilia constantly move the mucus layer to ward the mouth, where it

along with the trapped microorganism is swallowed and eliminated.

Besides, the action of coughing removes mucus that contains microorganisms. In the urethra rapid flow of urine washes away most microorganisms. Tear that wash the conjunctiva perform a similar defensive function.

Biochemical factors

These are chemical secretions produced by the body that inhibit microbial growth. The following are included as an example, keratin is a skin protein produced by the outher most cells of the skin, since it has very little water, the skin becomes very dry and therefore to most species of microorganism. The growth of microorganisms is inhibited in the gastrointestinal tract by hydrochloric acid and bile salt, which are secreted by the stomach and liver, respectively.

Lysozyme is an enzyme found in many body fluids and secretions such as tears. It can break down the cell wall of Gram-positive bacteria and a few gram-negative bacteria by hydrolyzing the peptidoglycan layer.

Complement is a family of more than twenty different proteins in serum that function as a non-specific defense against infection.

Interferons are small proteins produced by eucarytic cells in response to viral infection. The virally infected cell produces interferon for a few hours, even for a day, and it will excrete and used by other cells. When these cells become infected with the same or unrelated virus, the interferons cause the cells to produce molecules that prevent replication of the infecting virus.

Cellular mechanism

Alveolar macrophages like neutrophils and natural killers remove particles and organisms that enter the alveoli. Neutrophils are the first phagocytes in the infected area that can non-specifically phagocytize some microbes. Natural killer cells are large lymphocytes whose function is to kill undesirable cells such as tumor cells and virus infected cells.

Role of normal flora

The human body is inhabited by a large number of microorganisms, mainly bacteria, which together, are called the body's normal flora or commensals. The term normal flora implies that such microbial inhabitants are harmless For the most part, normal flora microorganisms do not cause disease.

The commensal can stop the growth of potentially pathogenic organisms through different mechanism such as occupying attachment sites and by producing substance against

pathogenic organism. They also compete for essential nutrients for their growth.

Inflammatory reactions

The inflammatory response is the vascular and cellular reaction to the presence of invading microorganisms or injury. It is one of the most effective defense mechanism in human and other animals. The process of inflammation may be divided in to the following stages:

- Initiation (Damage to tissue)
- Tissue response
- Leukocyte response
- Tissue repair (resolution)
- Cure.

The damaged cells at the site of injury initiate the tissue response by releasing chemical factors such histamine, which in turn trigger vasodilatation and increased permeability of capillaries, permitting influx of fluids and blood cells in to the site. Then, the phagocytic cells accomplish the leukocyte response, by engulfing the microbes and damaged tissue.

In addition to destroying and removing an injurious agent such as a microbe or its products, the inflammatory response also limits the effects of the agent or its products by confining it or walling it of from the surrounding tissues. This is possible

because blood clots around the site prevent the microbe or its products from spreading to the other part of the body.

The final stage of inflammation is tissue repair, when all harmful agents or substances have been removed or neutralized at the injury site. The ability of a tissue to repair it self depends on the part of the tissue involved. Skin, being a relatively simple tissue has a high capacity for regeneration. But nerve tissue in brain, appears not to regenerate.

2.1.2. Specific immunity

The specific immune response, also called acquired or adaptive immunity, is a defense system that protects the body against pathogenic microorganisms and other type of disease such as cancer. It allows the body to recognize, remember, and respond to a specific stimulus, an antigen. Specific immunity can result in the elimination of microorganisms and in the recovery from disease, and it frequently leaves the host with specific immunologic memory. This condition of memory or recall, acquired resistance, allows the host to respond more effectively if reinfection with the same microorganism occurs. As a consequence of such acquired immunity, we usually suffer from many diseases only once, for example measles. Specific immunity can be active or passive, and each of these types can in turn be naturally or artificially acquired.

Passive immunity

It is an immunity in which antibodies produced elsewhere are given to the individual. They are divided into two:

I. Naturally acquired passive immunity: refers to antibodies transferred from mother to fetus across the placenta and to the newborn in colostrums and breast milk during the first few months of life.

ii. Artificially acquired passive immunity: is introduction of antibodies that are formed by an animal or a human to an individual to prevent or treat infection.

Active immunity

It is a product of the individual's own immune system in response to a foreign antigen.

I. Naturally acquired active immunity: is immunity that comes from infections encountered in daily life.

ii. Artificially acquired active immunity: It is stimulated by initial exposure to specific foreign macromolecules through the use of vaccines to artificially establish a state of immunity.

2.3. Factors Associated With Immunologic Disease

Many factors appear to be responsible for differences in the immunity of individual's age; nutrition & genetic factor are some of them.

Age

Although non-specific and specific body defense are present in the unborn and newborn infants, many of these defenses are not completely developed in this group. There fore young children are at greater risk for disease. In older adults certain natural barriers to infection break don such as changes in the skin & in the lung weakening of specialized defenses against foreign invasion including the cough reflex.

In general, the ability to respond immunologically to disease is age related.

Nutrition

The importance of good nutrition to good health has always been emphasized. Good nutrition is known to be important to growth and development. The consequences of diet in many aspects of the immune response have been documented in multiple disorders. Every constituent of the body defense appears to influence by nutritional intake. Therefore a healthy

diet is important for maximum functioning of the immune system

Genetic factors

The possession of certain genes is linked to immune disorders these include genes that lead to a deficiency in the production of neutrophilis & complement.

Sickle cell disease predisposes to pneumococcal bacteraemia or Haemophilus influenza and E. coli infection.

Review Questions

1. What are the non-specific immunity
2. What are the steps involved in inflammatory reaction
3. Write the difference between active and passive immunity
4. Explain the factors that affect the immunity

CHAPTER THREE

THE LYMPHOID SYSTEM

Learning Objective

At the end of this chapter, students are expected to:

- Describe about primary and secondary lymphoid organ
- Explain about clonal selection theory
- Explain the difference between primary and secondary immune response

3.1 Lymphoid Tissue

The immune system is a network of cells and organs that extend through out the body and function as a defense against infection. The immune system has been recognized as a separate body system known as lymphoid system because its main cells are lymphocytes.

In mammalian immunologic development, the precursors of lymphocytes arise from progenitor cells of the yolk sac and liver. Later in fetal development and throughout the life cycle, the bone morrow becomes the main provider of undifferentiated progenitor cells, which can further develop in

to lymphoblast. Continued cellular development of lymphoid precursors and proliferation occurs as the cell travel to the primary and secondary lymphoid tissues.

3.1.1 The Primary Lymphoid Organ

The thymus and bone marrow are referred to a primary lymphoid organ because they provide conducive microenvironments that are essential for initial production of lymphocytes from progenitor cells.

The thymus is a gland situated in front of the heart and behind the sternum. Progenitor cells that leave the bone marrow migrate to the thymus for proliferation and differentiation. This process is facilitated by a hormone, thymosin. The differentiated cell is known as thymus- derived T cell.

Involution of the thymus is the first age-related change occurring in the immune system of humans. The thymus gradually loses up to 95% of its mass during the first fifty years of life. This will result in decreased synthesis of thymic hormone and loss of the ability to differentiate immature lymphocytes are reflected in an increased number of immature lymphocytes both with in the thymus and circulating peripheral blood T-cells.

Bone marrow is the source of progenitor cells. These cells can differentiate in to lymphocytes, granulocytes, erythrocytes, etc. The bone marrow also plays a role in the differentiation of progenitor cells into B-lymphocytes and functions as the bursa equivalent in human. It is from the term bursa, that the B-lymphocytes derived their name. Bursa of fabricius is the primary lymphoid organ in birds.

B-lymphocyte differentiations in the bone marrow continue through out lifetime. Mature lymphocytes that emerge form the thymus or bone marrow are in a “resting” state. They are mitotically inactive although they are potentially capable of undergoing cell division and of carrying out immunolgic functions, they are not yet been stimulated to do either when dispersed in to the blood stream, they are known as ‘naïve’ or ‘virgin’ lymphocytes.

3.1.2 Secondary Lymphoid Organ

The secondary lymphoid organs include lymph nodes, spleen, gut-associated lymphoid tissue, tonsils, blood and others, in to which the so-called ‘virgin’ lymphocytes migrates efficiently. The function of the secondary lymphoid organs is to maximize encounters between lymphocytes and foreign substances, and it is from this site that most immune responses are launched.

The relative percentage of T and B cells are different in different locations. The approximate percentage of lymphocytes in lymphoid organs is described in table 3.1.

Lymph nodes

Lymph nodes act like lymphoid filters in the lymphatic system. It responds to antigens introduced distantly and routed to them by afferent lymphatic. Generalized lymph node reactivity can occur following systemic antigen challenge.

Spleen

The spleen act like a lymphatic filter with in the blood vascular tree. It is an important site of antibody production in response to intravenous particulate antigen (e.g. bacterial). The spleen is also a major organ for the clearance of particles.

Gut – associated lymphoid tissue (GALT)

Gut-associated lymphoid tissue includes lymphoid tissue in the intestines (payer's patches) and the liver. Gut associated lymphoid tissue is involved in lymphocyte circulation, i.e. pre-B cells develop in payer's patches and after meeting antigen from the gut, they enter to the general circulation and then return back to the gut.

Tonsils

Tonsils are nodular aggregates of lymphoid tissues, their function is to detect and respond to pathogens in the respiratory secretion.

Blood

The blood is an important lymphoid organ and immunologic effector tissue. Circulating blood has enough mature T-cells to produce graft- versus- host reaction.

Table 3.1 approximate percentages of lymphocytes in lymphoid organs

Lymphoid organ	T-lymphocytes	B-lymphocytes
Thymus	100	0
Blood	80	20
Lymph nodes	60	40
Spleen	45	55
Bone marrow	10	90

Source: immunology & serology in laboratory medicine, page 64.

Most virgin lymphocytes have an inherently short life span and are programmed to die within a few days after leaving the marrow or thymus. How ever, if a cell receive signals that induce the presence of a specific foreign substance or pathogen, it may respond to it by a phenomenon known as

activation. In the process it may under go several successive cell divisions over a period of several day. Some of the resulting progeny cells then revert to the resting state to become memory lymphocytes (cells that resemble the virgin lymphocytes from which they are derived but which can survive for many years). The other progeny of an activated 'virgin' lymphocyte differentiate into effector cells, which survive for only a few days to carry out specific defensive activities against the foreign invader.

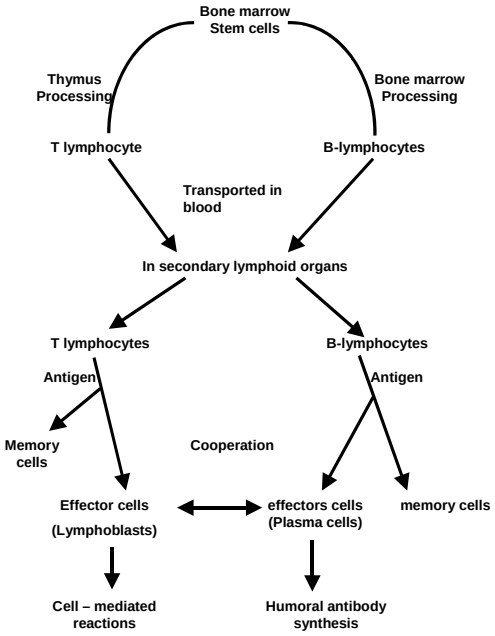


Figure 3.1 - Distinction between T lymphocytes and B-lymphocytes. Source: Principles of Microbiology, Ronald M-Atlas, 2nd edition, 1997 pp524.

3.2 The lymphocytes

The lymphocytes are one of the classes of white blood cells, capable of responding to antigens in specific host defense mechanism. Under the microscope, all lymphocytes look like, differing only in size and other physiologically variable features, yet under this surface of morphological homogeneity, there is a hidden homogeneity. First, the lymphocytes fall in to three large sets, the T, B. and natural killer (NK) cells. The NK cells constitute a special category; we will there fore leave them and focus on T and B cells. The T and B sets are splited in to subsets defined by a variety of criteria. Finally, each subset is a mosaic of clones, each clone express a specific receptor for a different antigenic eptiope.

3.2.1. Clonal Selection Theory

Lymphocytes have surface receptors specific for different antigens. As these cells differentiate and each of them expresses a particular receptor for gene, they are subsequently excluded form expressing all other receptor genes. The progeny of a given cell will therefore express the same gene and the same receptor. It will thus form a clone, a group of cells that are derived from the same ancestral cell. Since different cells express different receptors, the lymphocyte population of a vertebrate is a mosaic of store

selling shoes of all possible sizes. The customer entering the store (the antigen or epitope binding to the receptor) selects a particular pair of shoes (particular lymphocytes) and purchases it (stimulates the lymphocytes). The selected lymphocyte then divides and the progeny form a clone of identical specificity (clonal selection).

The fundamental property of lymphocytes is that all of the B-cell or T cell receptor proteins expressed by cells in a given clone are identical. All have precisely the same amino acid sequence.

The speed and intensity of response to a given antigen is determined largely by clonal selection. The larger the specific clone, the more lymphocytes are available that can recognize the antigen and can participate in the immune response. Burnet first postulated the principle of clonal selection, in the 1950s.

3.2.2. B-lymphocytes

B-cells represent less than 15% of the circulating lymphocytes and are derived from progenitor cells through an antigen independent maturation process occurring in the bone marrow and GALT. These cells serve as the primary source for cells

responsible for hurmonal (antibody) response, which is a primary host defense against microorganisms.

B- lymphocytes and humoral response

Participation of B-cells in the humoral immune response is accomplished by their stimulation in to plasma cells, with subsequent synthesis and secretion of immunogobulins after antigenic stimulation.

The humoral immune response; stimulation of B-cells to produce antibodies requires the interaction between macrophages, T-cells and B-cells. In order to recognize antigens, B-cells usually require the cooperation of antigen presenting cells. Macrophages are the major antigen presenting cells that phagocytize, process and present antigen to T-cells. The T-cell that contain an antigen on its surface bound with a specific antigen receptor of B-cell known as B-cell receptor as a result the B-cell recognize its specific antigen and it become activated.

Activation causes the selected B-cell to divide producing clone. It also causes the B-cells to differentiate further in to plasma cells, which produce the enormous quantity of antibodies needed to fight infection. Some members of this clone become effector cells that actively fight the current

infection while others remain as memory B-cells that are held in reserve to fight future infection by the same antigen.

Plasma cells are the end stage of B-cells differentiation, which are capable of synthesing and excreting antibodies. Plasma cells are not found normally in the circulating blood.

Primary and secondary humoral immune response

B cells can be stimulated in their resting state to enlarge, divide, mature and secrete antibody. The proper signal for this sequence depend on the type of antigen (foreign material) i.e. exposure of antigen for the first time or second exposure. Depending on the type of espouser the immune response also vary.

Primary immune response

The production of antibody during a primary immune response occurs when a person first encounters a particular antigen. The type of antibody first appear are of the IgM class. The production of detectable serum IgM may take 1-2 weeks the produced antibody will decline with in short period.

Secondary immune response

Secondary immune response is initiated by memory B-cells when there is second exposure with similar antigen. IgG is the major antibody produced in secondary immune response. The

production of this antibody in detectable amount may take very short time as compared to the primary response. The IgG may persist for many months or years. The secondary immune response has high affinity than primary immune response because of the memory B-cells.

Review Questions

1. Write the site that B-cell and T-cell proliferation takes place
2. Describe the function of secondary lymphoid organs.
3. Discuss what clonal selection mean
4. Describe the difference between primary and secondary humoral immune response.

CHAPTER FOUR

ANTIGENS, ANTIBODIES AND THE COMPLEMENT SYSTEM

Learning Objectives

At the end of this chapter, students are expected to:

- Describe the characteristics of antigens and antibodies
- Explain the different classes of immunoglobulin
- Describe the two path way of complement activation
- Discuss the role of complements in immune response

4.1 Antigens

Antigens are substances that are recognized by a particular immunoglobulin or T-cell receptor and they can serve as the target of an immune response.

Immunogens are any substance that is capable of inducing an immune response.

Antigenic determinant or epitope is the specific site to which a particular immunologlobulin or T-cell receptor binds.

Many immunogens, including all microbial pathogens, are complex assemblages containing several different types of molecules, not all of which are antigenic.

Not all antigens are immunogenic i.e. not every chemical substance that can be bound by an immunoglobulin is capable of inducing an immune response.

Antigenic molecules may be multivalent, having multiple epitope, or monovalent, having only one epitope. Generally, multivalent antigens produce a stronger immune response than monovalent antigens because wide arrays of antibody molecules are made against the multiple antigens.

Adjuvants

The response to an immunogen is often enhanced if it is administered as a mixture with substances called adjuvants. Adjuvants function in one or more of the following ways;

- By prolonging retention of the immunogen,
- By increasing the effective size of the immunogen,
- By stimulating the local influx of macrophages and/or other immune cell types to the injection site and promoting their subsequent activities.

The most widely used adjuvant for humans is alum precipitate, a suspension of aluminum hydroxide on to which the immunogen is adsorbed. This adjuvant increases the

effective size of the immunogen and so promotes its ingestion and presentation by macrophages.

For a substance or molecule to be immunogenic certain conditions must be fulfilled; these are: chemical composition, molecular size, etc.

Chemical composition

Large, macromolecular proteins are the most potent immunogens. Polysaccharides and short polypeptides can also be immunogenic under certain circumstances. Pure lipids and nucleic acids have not been shown to be immunogenic, thus they can be an example of molecules that are antigenic but not immunogenic

Molecular Size

Extremely small molecules such as amino acids or monosaccharides are usually not immunogenic implying that a certain minimum size is necessary for immunogenicity. A few substances with molecular weights below 1000 have proven to be immunogenic, but as a rule molecules with molecular weights below 10,000 are only weakly immunogenic or not immunogenic at all. The most potent immunogens are proteins with molecular weight, greater than 100,000.

Chemical Complexity

A molecule must possess a certain degree of chemical complexity to be immunogenic. For instance, simple polypeptides that contain tyrosine are better immunogens than are comparable polypeptides with out tyrosine, and immunogenicity of such polymers is directly proportionate to their tyrosine contents.

Foreignness

The immune system normally discriminates between self and nonself, so that only molecules that are foreign to the host are immunogenic. Hence, albumin isolated from the serum of a rabbit and injected back in to the same or another rabbit will not yield an immune response. Every rabbit is tolerant to this endogenous protein. Yet the same protein, if injected in to other vertebrate species, is likely to evoke substantial antibody responses.

Method of administration

Whether a substance will evoke an immune response also depends on the dose and mode of administration. A quantity of substance that has no effect when injected intravenously may evoke antibody response when injected subcutaneous particularly if it is accompanied by an adjuvant.

4.2. Antibodies

Antibodies are glycoproteins, which are sensitized, and secreted by plasma cells in response to specific antigenic stimulation and it forms about 20% of plasma protein.

Many antibodies can be isolated in the gamma globulin fraction of protein by electrophoresis separation. The term immunoglobulin, however, has replaced gamma globulin because not all antibodies have gamma electrophoresis mobility. Antibodies can be found in blood plasma or serum and in many body fluids such as tears, saliva and colostrums.

The primary function of an antibody in body defenses is to combine with antigen, which may be enough to neutralize bacterial toxins or some viruses. A secondary interaction of an antibody molecule with another effector agent such as complement is usually required to dispose of larger antigens such as bacteria.

There are two types of antibodies: complete and incomplete antibodies. **Complete antibodies:** are antibodies which are heat resistant, when they combine with its specific antigen they will produce different immunologic reaction. These antibodies are capable of passing the transplacental barrier.

Incomplete antibodies: Some times known as blocking antibodies these are heat labile substances that do not show any immunologic reaction when they bind with an antigen. Incomplete antibodies are not able to cross the placental barrier.

Antibodies are generally described in terms of their reactions with antigen. These include:

- Antitoxin- antibodies to toxins or toxoids, which neutralize the antigen.
- Agglutinin – antibodies which first immobilize motile bacteria and aggregate cells forming clumps.
- Precipitins – antibodies, which form complexes with soluble antigens forming precipitates.
- Lysine – antibodies, which together with complement dissolve the antigenic cells.
- Opsonins – antibodies, which combine with, surface components of microbial and other cells so that they are more readily phagocytized.

4.3. Immunoglobulins

4.3.1 Basic Structure of an Immunoglobulin

Immunoglobulins are made up polypeptide chain held together by disulphide bond. Each half of the molecule consists of one heavy (long) chain and one light (short) chain.

The heavy (H) chain is roughly twice as large as the light (L) chain. Therefore a monomer molecule of immunoglobulin is composed of four polypeptide chain; a pair of two identical H and L chain. Every immunoglobulin contains equal number of heavy and light chain and can be represented by the general formula $(H_2L_2)_n$.

All of the light chains and all of the heavy chains in any single immunoglobulin protein are identical. In both heavy and light chains, this variability is confined largely to the N-terminal; where as the sequence of the other domains remain relatively constant. For this reason, the N-terminal in a heavy or light chain polypeptide is called the variable region V^H and V^L, respectively. The other domains are collectively termed as the constant region C_H and C_L.

Light chain polypeptide contain only a single C_L domain but heavy chain C_H regions comprise 3 or more domain which are numbered sequentially (CH1, CH2, --- etc) beginning with the domain closest to V_H. In overall, the protein has a T or Y shaped configuration when viewed schematically. The region at the base of each arm in the T or Y located between the CH, and CH_2 domain is called the hinge region in most immunoglobulin. The hinge region confers flexibility, enabling the two arms to move relatively freely with respect to each other.

Types of light chain

All light chains have protein with molecular weight of about 23,000 and can be classified in to two distinct types, kappa (k) and lambda (λ), on the basis of their constant light region sequence. There is no known functional difference between these two types. A given immunoglobulin molecule always contains exclusively either k or λ chains never a mixture. Similarly, any given B-lineage cell produces only one type of light chain.

The proportion of K to λ chain in the entire population of immunoglobulin in an individual is about 2:1 but it may vary from species to species.

Types of heavy chain

Human express five different classes of immunoglobulin heavy chains, which differ considerably in their constant heavy region, which in turn result difference in their physical and biologic properties. All of the heavy chains in a given immunoglobulin molecule are identical. The heavy chain polypeptides range in molecular weight from about 50,000-70,000.

The classes of heavy chains are designated as μ, δ, α, γ and Σ. The immunoglobulins that contain these heavy chains are designated as IgM, IgD, IgG, IgA & IgE, classes, respectively.

On the basis of relative minor difference in constant heavy region, they are sub divided as γ1,2,3,4, α1 and α_2. The corresponding immunoglobulin subclasses are denoted as IgG, IgG_2, IgG_3, IgG_4, IgA_1, & IgA_2. Normal individuals express all these nine classes and subclasses because each is encoded by a separate genetic locus and inherited independently.

4.3.2 Function of Different Regions of Immunoglobulins

Immunoglobulin chains are cleaved by proteolytic enzyme like papain. These enzymes split an immunoglobulin molecule in to three fragments of roughly similar size.

Two of the fragments are identical to one another and each consists of an entire light chain, the variable heavy and constant heavy (CH^1) domains of one heavy chain, these fragments thus contain the antigen binding sites of the protein called antigen- binding fragment (Fab fragments). Each basic four-chain unit contains two separate but identical antigen binding sites called divalent.

The third fragment comprises the carboxyl terminal portions of both heavy chains held together by disulfide bond, it is designated by crystallizable or Fc fragment. The structure of

this third fragment is identical for many different immunoglobulin molecules.

Most of the secondary biologic properties of immunoglobulin such as the ability to activate complement are determined by sequence in the Fc region of the protein. This is also the region that is recognized by the Fc receptors found on many types of cells.

4.3.3 Classes of Immunoglobulins

Five distinct classes of immunoglobulin molecules are recognized in most higher mammals: IgG, IgM, IgA, IgD and IgE. These immunoglobulin classes differ from each other in characteristics such as molecular weight, sedimentation coefficient between classes, the immunoglobulins vary with in each class.

Immunoglobulin G (IgG)

The major immunoglobulin in normal serum is IgG. It accounts approximately 75% of the total serum immunoglobulin. An IgG molecule consists of two light chain and two heavy chains linked by disulphide bonds. Because it has two identical antigens binding site it is said to be divalent. This immunoglobulin diffuses more readily than other immunoglobulins in to the extra vascular spaces, and it

neutralizes toxins and binds to microorganisms in to the extra vascular spaces. It is the only class of immunoglobulin that can cross the placenta, and it is responsible for protection of the newborn during the first months of life. In addition, when IgG complexes are formed, complement can be activated.

Subclasses of IgG

There are four subclasses of immunoglobulin molecule namely IgG_1, IgG_2, IgG_3 & IgG_4. These subclasses differ in their heavy chain composition and in some of their characteristics such as biologic activities.

Table 4-1 characteristics of IgG subclasses

	IgG1	IgG_2	IgG_3	IgG4
Percentage of serum	65	24	7	4
Complement fixation	4^+	2^+	4^+	+
Half-life (days)	23	23	8	23
Placental Passage	+	?	+	+

Adapted from Mary Louis Turgeon- Basic immunology and serology in laboratory medicine .2nded. Pp19.

Immunoglobulin M (IgM)

IgM constitutes approximately 10% of normal serum immunoglobulin. It is the largest of all immunoglobulins, which

consists of five monomers joined together by a J-chain in to a pentamer. It has ten antigen binding sites. This antibody is produced early in an immune response and is largely confined intravascularly because of its large size. IgM is effective in agglutination and cytolysis reactions. In human it is found in smaller concentrations than IgG and IgA.

Immunoglobulin A (IgA)

IgA represents 15% to 20% of the total circulatory Immunoglobulin pool. It is the predominant immunoglobulin in secretions such as tears, saliva, colostrums, milk, and intestinal secretions. IgA is synthesized largely by plasma cells located on body surface. If cells in the intestinal wall produce the IgA, it may pass directly into the intestinal lumen or diffuse into the blood circulation. As IgA is transported through intestinal epithelial cells or hepatocytes, it binds to a glycoprotein called the secretary piece; it protects IgA from digestion by gastrointestinal proteolytic enzymes and forms a complex molecule named secretary IgA. Secretary IgA is of critical importance in protecting body surfaces against invading microorganisms. It provides external surface of the body with protection form microorganisms. IgA has two subclasses (IgA_1 and IgA_2), which are expressed in a 5:l ratio in the blood and have similar properties. Unlike IgG, IgA do not activate complement and unable to cross the placental barrier.

Immunoglobulin D (IgD)

IgD is a monomer, which constitutes less than 1% of the total immunoglobulin pool. It is very susceptible to proteolysis and is primarily a cell membrane immunoglobulin found on the surface of B-lymphocytes in association with IgM.

Immunoglobulin E (IgE)

IgE is a monomer, which occurs, in minute quantity, 0.004%, in the blood plasma of parasitized individuals. IgE is of major importance because it mediate some types of hypersensitivity (allergic) reactions and generally responsible for an individual's immunity to invading parasites. The IgE molecule is unique in that it binds strongly to a receptor on mast cells and basophiles. Together with antigen, mediates the release of histamines and heparin from these cells.

Table 4.2 properties of human immunoglobulins

	IgG	IgA	IgM	IgD	IgE
Heavy chain classes	γ	α	μ	δ	Σ
Heavy chain subclasses	γ1,2,3,4	α_1,α_2	-	-	-
Light chain class	6-7	7	19	7-8	8
Molecular weight x1000	150	160	900	180	190
Complement fixation	+	0	++++	0	0
Serum concentration approximate (mg/d/)	1000	200	120	3	0.05
Serum half life (days)	23	6	5	3	2
Placental transfer	+	0	0	0	0

Adapted from, Daniel P.stites, et al Basic & Clinical immunology 8th ed.pp71

4.4 Complement System

The immune system is composed of a large and complex set of widely distributed elements. The specific recognition system is ultimately composed of receptors on T and B-lymphocytes the only specific components of the immune mechanism. The immune system also has a nonspecific effecter mechanism that usually amplifies the specific functions. The nonspecific features include the complement system. The complement system is a heat-labile series of more than 18 plasma proteins. Normally, these proteins are in

an inactive form, but specific signal can activate the first protein of the team.

4.4.1 Complement Activation

Activation of complement system can be initiated by either an antigen antibody complex or by variety of foreign surfaces; there are two major pathways for complement activation the classical and alternate pathway.

Classic path way

The principal component of the classic path way is C_1 through C_9. The sequence of complement activation does not follow the expected numerical order. The sequence is $C_{1,4,2,3,5,6,7,8, \& 9.}$

C_3 is present in the plasma in the largest quantities; fixation of C_3 is the major quantitative reaction of the complement cascade.

The classic path way is initiated by the complexing of antigen to its specific antibody, either IgM or IgG, and is the primary amplifier of the biologic effects of humoral immunity.

The classic pathway is composed of three stages:

1. Recognition
2. Enzymatic activation
3. Membrane attack leading to cellular destruction

The recognition unit of the complement system is the Cl complex Clq, Clr and Cls, an interlocking enzyme system. The Cl complex is a unique feature of the classic pathway subcomponent binds directly to an immunoglobulin molecule. The other two subcomponents, Clr and Cls, do not bind to the immunoglobulin but are involved in subsequent activation of the classic pathway.

A single IgM molecule is potentially able to fix Cl, but at least a pair of IgG molecules is required for this purpose.

Clr and Cls activate each other and in turn, $C_4$and C_2, splitting each into two fragments. One of the C_4 fragments and one of the C_2 fragments combine to formC_3 convertase (C_4b2b)& activateC3, cleaving in to fragments called C_3a and C_3b. C_3a is a powerful opsonin and C_3b is an anapylatoxin. C_3b forms a complex with C_{4b2b}, producing a new enzyme, C_5 convertase, which cleaves C_5 chemo tactic factors. C_{5b} binds to C_6 and C_7 to form a complex that insert into the membrane bilayer. C_8 then binds to the $C_{5b}/C_6/C_7$ complex followed by the polymerization of up to 16_{C9} molecules to produce the membrane attack complex that causes cytolysis.

Alternative Pathway

Alternate pathway is a non-antibody initiated pathway. Microbial and mammalian cells surface can activate the

alternate pathway in the absence of specific antigen antibody complexes. The alternate pathway shows points of similarity with the classic sequence. Both pathways generate a C_3 convertase that activate C_3 to provide the pivotal event in the final common pathway of both systems.

A key feature of the alternate pathway is that the first three proteins of the classic activation pathway C_1, C_4, and C_2 do not participate in the cascade sequence. C_3 component is activated by means of complement proteins: factor B, which resembles C_2; factor D, similar with C_1 and properdin.

The activator of the alternate pathway, properdin, catalyzes the activation of C_3. The uptake of factor B on to C_{3b} occurs when C_{3b} is bound to an activator surface. C_{3b} and factor B combine to from C_{3b}, B, which is converted into an active C_3 convertase, $C_{3b}B$, Which is converted in to an active C_3 convertase. C3b, Bb. This result from the loss of a small fragment, Ba, through the action of the enzyme, factor D. The C3b, Bb complex is able to convert more C3 to C3b, which binds more factor B.

C3b, Bb complex decays due to the loss of Bb with a half-life of approximately five minutes. However, if properdin (P) binds to C3b, Bb, forming C3b, Bbp, and the half-life is extended to 30 minutes.

The association of numerous C3b units, factor Bb and properdin on the surface of an aggregate of protein on the surface of a microorganism has a potent activity as a C5 convertase with the cleavage of C5, the remainder of the complement cascade continues as in the classic pathway.

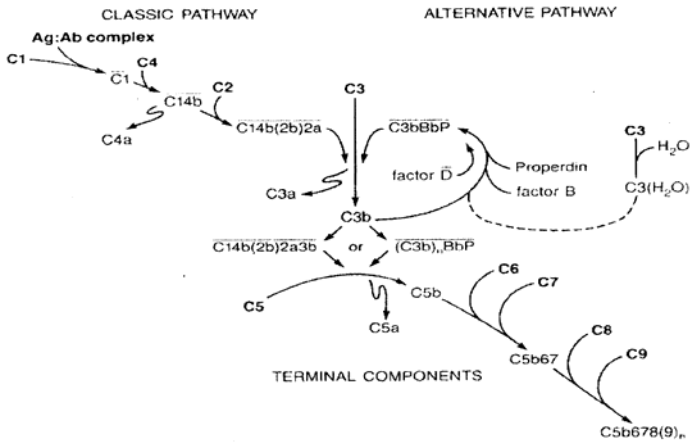


Fig 4.2 the complement cascade
Adapted from Daniel P. Stites, et al. Basic and clinical Immunology, page 125.

4.4.2 The Role of Activated Complement in Immune Response

The activation of complement and the product formed during the complement cascade have a variety of physiologic and

cellular consequences. Physiologic consequence includes blood vessel dilation and increased vascular permeability.

The cellular consequences include the following:

1. Cell activation such as the production of inflammatory mediators. Eg.C3a, C4a& C5a can produce degranulation of mast cells with release of inflammatory mediator.
2. Cytolysis or haemolysis, if the cells are erythrocytes. Insertion of the C_{5b6789} complex into the cell surface leads to killing or lysis of many types of cells including erythrocytes.
3. Opsonization, C3b complement is a powerful opsonin, which renders cells vulnerable to phagocytosis.

4.4.3. Properties of Complements

Heating at 56^{o}c for 30 minutes can inactivate complements. Aging, especially at room temperature leads to almost complete deterioration within a day or two and even in the refrigerator, considerable loss of activity occurs in 3-4 days. Complement may be destroyed by violet shaking for 20-25 minutes, by some acids and alkalis; ether and soaps. As a result all glassware must be chemically clean, although not necessarily sterile. Complements may be preserved for long periods by freezing and storing at low temperature e.g. lyophilization.

Review Questions

1. Describe the difference between antigen and immunogen
2. Discuss the characteristics of antibodies
3. Discuss the five classes of immunoglobulin
4. Explain how complement proteins are activated in both classic and alternate pathway.
5. Write the major effect of complement activation

CHAPTER FIVE

THE CELLULAR IMMUNITY

Learning Objectives

At the end of this chapter, students are expected to:

- Explain the cell-mediated response and its regulation
- Discuss about delayed hypersensitivity reaction and autoimmune diseases

5.0. Cellular Immunity

Cell mediated immunity consists of immune activities that differ from antibody- mediated immunity. It is moderated by the link between T-lymphocytes and phagocytic cells.

T-lymphocytes do not directly recognize the antigens of microorganisms or other living cells, but rather when the antigen is present on the surface of an antigen- presenting cell, the macrophages. Lymphocytes are immunologically active through various types of direct cell-to-cell contact and by the production of soluble factors.

Cell mediated immunity is responsible for the following immunologic events:

- ✓ Immunity to intracellular organisms
- ✓ Rejection of foreign tissue grafts
- ✓ Immunity to viral and fungal antigens
- ✓ Delayed hypersensitivity

5.1 Cell- Mediated Response

Sixty to eighty percent of the total circulating lymphocytes are T cells derived from progenitor cells that mature in the thymus gland under the influence of thymic hormones. These cells are responsible for cellular immune responses and are involved in the regulation of antibody reactions either by helping or suppressing the activation of B-lymphocytes.

There are at least three functionally distinct types of T cells: cytotoxic or effector T cells, helper or regulatory T cells, suppressor T cells

Cytotoxic T cells: are effectors cells, found in the peripheral blood, that have the capacity to kill other cells. These cells can destroy virally infected cells.

Helper T cells: secret a variety of substances that help B cells make antibody response, stimulate activated T cells to

proliferate, and activate macrophages. T helper cells control many B cell functions, including proliferation and differentiation.

Suppressor T cells: are thought mainly to inhibit the response of helper T cells. T suppressor cells are capable of suppressing a variety of T cell functions such as cytotoxic response, and B cell responses such as suppression or T helper cells or antibody synthesis by plasma cells.

All T-cells have antigen receptor protein (T cell receptor) with which they bind foreign antigens. In order to recognize a foreign antigen, T- cells must simultaneously recognize particular types of self-antigens, which are structural components of the surface of human cells. These antigens don't normally stimulate a destructive immune response.

In summary, for T-cell antigen recognition, a foreign antigen is taking up and processed by antigen presenting cells usually macrophages and then the processed antigen is combined with a self antigen to form antigen complex.

The antigen-binding site of a T-cell must simultaneously recognize and bind both a foreign and a self-antigen. The self-antigen is known as major histocompatability complex (MHC), it has two major classes:

Class I major histocompatability complex antigen present on all nucleated cells of the body, it is recognized by only T cytotoxic cells. Class II major histocompatability complex antigen, present only on macrophages, it recognized by only T-helper cells.

The first cell to be activated in any immune response is the T helper cell. Activation of T helper cell is enhanced by some factors produced by macrophages. The stimulated T helper cell stimulates it self and proliferate in increased amount. This activated T helper cell also helps to stimulate T cytotoxic cells. When these cells recognize antigen presented in combination with the class I major histocompatability antigen.

Activated T cells respond with direct cytotoxic killing (T cytotoxic cells) or with immune regulation either by intensifying the immune response (T helper cells) or by lowering the immune response (T suppresser cells).

Helper and suppressor T cells are the principal regulators of immune responses. Sensitized T cells protect the human body against infection by mediating intracellular pathogens that are viral, bacterial, fungal, or protozoal. In addition, T cells are responsible for chronic rejection in organ transplantation.

5.2 Delayed Type of Hypersensitivity

Cell- mediated immunity consists of immune activates that differ from antibody mediated immunity. Cell-mediated immunity is moderated by the link between T lymphocytes and macrophages. T cells do not recognize the antigen of microorganisms or other living cells but are immunolgically active through various types of direct cell-to-cell contact and by the production of soluble factors. The delayed type reaction is cells mediated and involves antigen sensitized T cells, which respond directly or by the release of lymphokines to exhibit contact dermatitis and allergies of infection. Delayed hypersensitivity reaction is one of cell mediated immunity immunologic events e.g. Tuberculin test.

5.3 Auto Immune Disease

Autoimmunity represents a breakdown of the immune systems ability to discriminate between self and nonself. Autoimmune diseases are characterized by the persistent activation of immunologic effectors mechanism that alter the function and integrity of individual cells and organs. The sites of organ or tissue damage depend on the location of the immune reaction.

In autoimmune disease, immunoglobulins (autoantibodies) or cytotoxic T cells display specificity for self-antigens or auto antigens and contribute to the pathogenesis of the disease. The potential for autoimmunity, if given appropriate circumstances, is constantly present in every immunocompetent individual because lymphocytes that are potentially reactive with self- antigens exist in the body. The development of autoimmunity is influenced by different factors these include; genetic factors, Age and exogenous factors.

Genetic factors

Although a direct genetic etiology has not been established in autoimmune disease, there is a tendency for familial aggregates to occur. E.g. the presence of certain human leukocyte antigen is associated with an increased risk of certain autoimmune state. Another factor related to genetic inheritance is that autoimmune disorders and autoantibodies are found more frequently in women than in men.

Age: Autoantibodies are manifested infrequently in the general population. The incidence of autoantibodies, however increase steadily with age, reaching a peak at around 60 to 70 years.

Exogenous factors: Ultraviolet radiation, drugs, viruses, and chronic infectious disease may all play a role in the

development of autoimmune disorders. These factors may alter antigens, which the body then perceives as non-self.

The normal functioning of immunolgic regulatory mechanisms usually prevents autoimmune disease. When these controls dysfunction antibodies to self antigens may be produced and bind to antigens in the circulation to form circulating immune complexes or to antigens deposited in specific tissue sites.

Some of the examples of autoimmune disease are Addison disease, pernicious anemia, Rheumatoid arthritis, and systemic Lupus erythematosus.

Review Questions

1. What are three major types of T-cells?
2. Describe the function of each T-cells type
3. Explain how activation of cell mediated immunity takes place
4. Discuss the different factors that influence the development of autoimmune disease.

CHAPTER SIX

ANTIGEN ANTIBODY INTERACTIONS

Learning Objectives

At the end of this chapter, students are expected to:

- Describe the different immunolgic reaction and their role in the diagnosis of disease.
- Explain factors that affect the antigen antibody reactions

6.1 Principle of Antigen Antibody Interactions

In the field of immunology, many serologic techniques are used to detect the interaction of antigens with antibodies. These methods are suitable for the detection and quantitation of antibodies to infectious agents, as well as microbial and non-microbial antigens.

In antigen antibody interaction determination of either antigen or antibody is possible, this determination follows a general principle: know antigen suspension or antiserum is used to detect and measure unknown antibody or microbial antigen.

6.2 In Vitro Antigen Antibody Reactions and Their Role in the Diagnosis of Disease

There are different types of antigen antibody interaction these include

- Precipitation reaction
- Agglutination reaction
- Complement fixation reaction
- Enzyme Immuno Assay (EIA)
- Radio Immuno Assay (RIA)

6.2.1 Precipitation Reaction

Precipitins can be produced against most proteins and some carbohydrates and carbohydrates- lipid complexes. Various system are available in which precipitation tests are performed in semisolid media such as agar or agarose, or nongel support medium such as cellulose acetate. Agar has been found to interfere with the migration of charged particles and has been largely replaced as an immunodiffusion medium by agarose. Agarose is a transparent, colorless, neutral gel. In the clinical laboratory several applications of the precipitation reaction are used. These methods include:

- Immunodiffusion
- Electroimmunodiffusion

Immunodiffusion: These are of two type: single and double immuodiffusion.

I. Double diffusion

This technique also referred to as the Ouchterlony method, may be used to determine the relation ship between antigen and antibodies.

Principle: Antibody dilutions and specific soluble antigens are placed in adjacent wells. If the well size and shape, distance between wells, temperature, and incubation time are optimal, these solutions diffuse out, bind to each other, cross-link, and form a visible precipitate at the point of equivalence perpendicular to the axis line between the wells the precipitation bands will be compared with a standard antigen. The precise location of the band depends on the concentration and rate of diffusion of antigen and antibody. In a condition of antibody excess, the band will be located nearer the antigen well. If two antigens are present in the solution that can be recognized by the antibody, two precipitin bands form independently.

Antibodies associated with autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus can be identified by double diffusion.

Identity

An identity reaction is indicated when the precipitin band forms a single smooth area. This precipitin is formed between the antibody and the two test antigens fuses (figure 6-1A), indicating that the antibody is precipitating identical antigen specificities in each preparation. This does not mean that the antigens are necessarily identical; they are only identical insofar as the antibody can distinguish the difference.

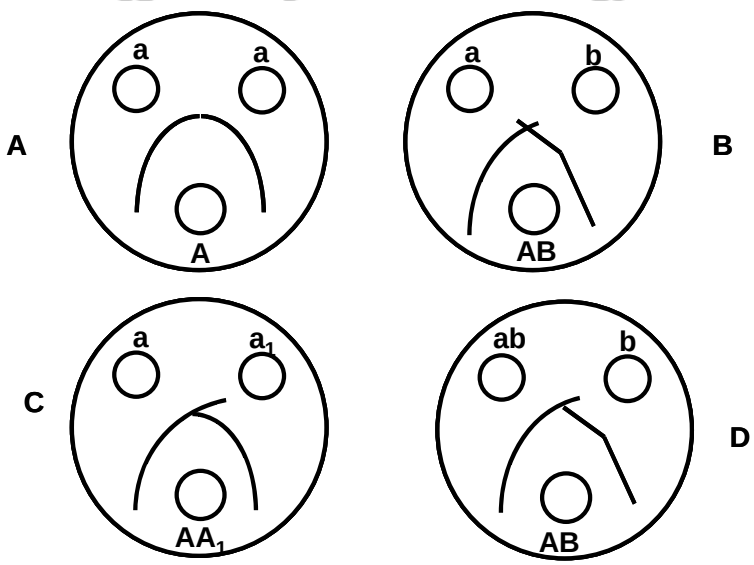


Figure 6-1

Precipitation pattern of ouchterlony type of immunodiffusion.
(From Mary LT: Immunology and serology in laboratory medicine, 2nd ed,st Louis, 1996,Mosby)

Nonidentity

A non-identity pattern (Fig 6-1B) is expressed when the precipitation line cross each other. They intersect or cross because the sample contain no antigenic determinants in common.

Partial Identity

In a partial identity pattern (Fig 6-1C), the precipitation lines merge with spur formation. This merger indicated that the antigen are non identical but possess common determinants.

II. Single radial immunodiffusion

This is a simple and specific method for identification and quantitation of a number of proteins found in human serum and other body fluids.

Principle: Radial immunodiffusion is based on a technique using a precipitin reaction in which specific antibody is added to a buffered agarose medium, serum containing the test antigen is placed in a well centered in the agarose. The diameter of the resulting precipitin zone is related to the concentration of antigen placed in a well.

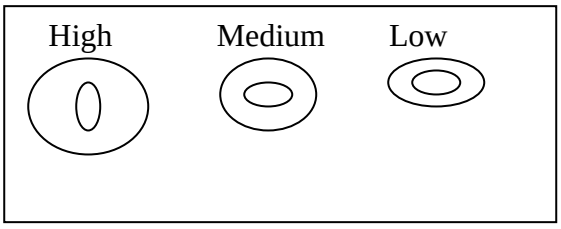


Figure 6.2 single radial immunodiffusion.
The unknown antigen is in the wells and the specific antibody in the agar, the greater the antigen concentration the larger the circle of precipitation.

Eletroimmuno diffusion (EID)

EID is a variation of the double immunodiffusion reaction in a support medium such as cellulose acetate or agarose through the use of an electric current that enhances the mobility of reactants and increase their movement towards each other.

Antibody is placed in the well favoring its migration in the direction of the cathode; antigens that tend to be more negatively charged and placed in the well that favors migration of the anode. Precipitin bands form at a point of equivalence in a shorter periods of time.

Electro immunodiffusion method, like immunodiffusion procedures, are classified into one-or-two-dimensional, singles, or double diffusion when a voltage is applied across

the gels to move the antigens and antibodies together, immuno-double diffusion becomes counter current immuno electrophoresis (CIE) radial immunodiffusion (RID) becomes electro immunoassay (EIA).

Counter Current immunoelectrophoresis (CIE) is a variation of the classic precipitin procedure; it merely adds an electrical current to help antigens and antibodies move to wards each other more quickly than in simple diffusion. The procedure takes advantage of the net electric charge of the antigens and antibodies being tested in a particular test buffer. Variables such as types of gel, amount of current, a concentration of antigen and antibody must be carefully controlled for maximum reactivity. The sensitivity of CIE is 10 to 20 times greater than in immuno-double diffusion, however, it is more expensive than other techniques such as immunodiffusion

Electro immunoassay

Antigens may be quantitiated by electrophoresis than in an antibody-containing gel electroimmunoassay. This technique combines the speed of electrophoresis with the accuracy and sensitivity of radioimmunoassay. .

6.2.2 Agglutination Reaction

Precipitation and agglutination are the visible expression of the aggregation of antigens and antibodies through the formation of a framework in which antigen particles or molecules alternate with antibody molecules.

Agglutination of particles to which soluble antigen has been absorbed produces a serum method of demonstrating precipitins. Example of artificial carriers includes latex particles and colloidal charcoal. Cells unrelated to the antigen, such as erythrocytes coated with antigen in a constant amount can be used as a biologic carriers. Whole bacterial cells can contain an antigen that will bind with antibodies produced in response to that antigen when it was introduced into the host.

Agglutination tests are easy to perform and in some cases are the most sensitive tests currently available. These tests have a wide range of applications in the clinical diagnosis of noninfectious immune disorders and infectious diseases.

Latex agglutination

In latex agglutination procedures, antibody molecules can be bound to the surface of latex beads. Many antibody molecules can be bound to each latex particle, increasing the potential

number of exposed antigen-binding sites. If an antigen is present in a test specimen, the antigen will bind to the combining sites of the antibody exposed on the surface of the latex heads, forming visible cross-linked aggregates of latex beads and antigen. In some test systems, latex particles can be coated with antigen. In the presence of serum antibodies, these particles agglutinate in to large visible clumps.

Examples of tests based on latex agglutination reaction include C-reactive protein, IgG rheumatoid factors, and IgM rheumatoid factors.

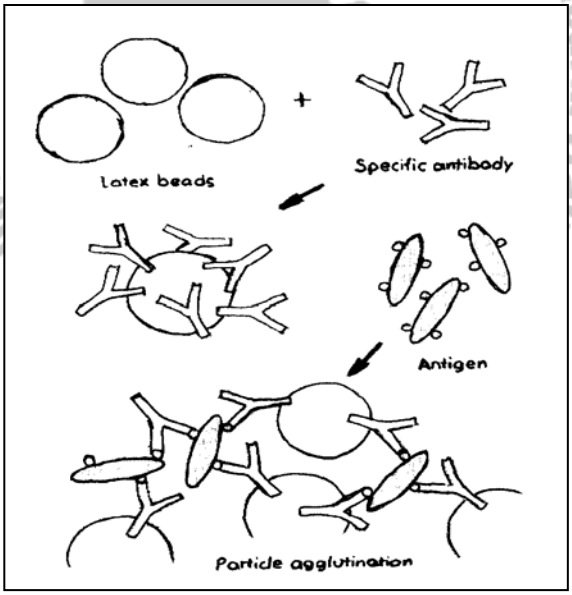


Figure 6.3 Latex agglutination reaction

Direct bacterial agglutination

Direct whole pathogens can be used to detect antibodies directed against pathogens. The most basic tests are those that measure the antibody produced by the host to determinants on the surface of a bacterial agent in response to infection with that bacterium. In a thick suspension of the bacteria, the binding of specific antibodies to surface antigens of the bacteria causes the bacteria to clump together in visible aggregates. This type agglutination is called bacterial agglutination. Because tube testing allows more time for antigen-antibody reaction, it is considered to be more sensitive than slide testing.

Indirect or passive hemagglutination

Hemagglutination is agglutination of red blood cells, and tests for antibody detection. In the indirect or passive hemagglutination technique, erythrocytes are coated with substances such as extracts of bacterial cells, protozoa or purified polysaccharides or proteins.

Erythrocyte of animals such as sheep or rabbits, or from group “O” humans, function as carrier for detecting and titrating the corresponding antibodies by agglutination. This technique is called indirect or passive hemagglutination testing because it is not the antigen of the erythrocytes

themselves but the passively attached antigens that are bound by antibody .For example, in rubella antibody test, erythrocytes are coated with rubella antigen. In the presence of antibody, agglutination occurs.

Hemagglutination Inhibition technique

Hemagglutination inhibition test is used to detect some viral antibodies, for example, rubella. A known quantity of rubella viral antigen is mixed with dilutions of the patient's serum, to which red blood cells are added. If the serum lucks antibody, the virus will spontaneously attach to the red cells, link together, and agglutinate. If antibody to the virus is present, all of the virus particles will be bound by antibody, which prevents or inhibits hemagglutination. The serum is therefore positive for the antibodies. The highest dilution of serum that totally inhibits agglutination of red cells determines the antibody titer of the serum.

Disadvantage of this technique include: time consuming, & subjective bias in the interpretation for results. Negative results do not always indicate the absence of antibody. In some case false negative results can occur from a low titer of antibody. Nonspecific inhibitors can cause false positive results.

6.2.3 Complement Fixation Reaction

Complement fixation is a classic method for demonstrating the presence of antibody in serum. This method consists of two components. The first component is an indicator system consisting of a combination of sheep red cells; complement-fixing antibody produced against the sheep red cells in another animal, and an exogenous source of complement, usually guinea pig serum. When these three components are combined in an optimal concentration, the antisheep cell antibody, hemolysin, can bind to the surface of the red cells. Complement can subsequently bind to this antigen antibody complex and cause cell lysis. The second component consists of a known antigen and patient serum, which are added to a suspension of sheep erythrocytes, hemolysin, and a complement.

The two components of the complement fixation procedure are tested in sequence. Patient serum is first added to the known antigen, and complement is added to the solution. If the serum contains antibody to the antigen, the resulting antigen antibody complexes will bind all of the complement. Sheep red cells and hemolysin are then added. If complement has not been bound by an antigen antibody complex formed from the patient serum and known antigen, it is available to bind to the indicator system of indicates both a lack of

antibody and a negative complement fixation test. If the patient's serum does contain a complement fixing antibody appositive result will be demonstrated by the lack of haemolysis.

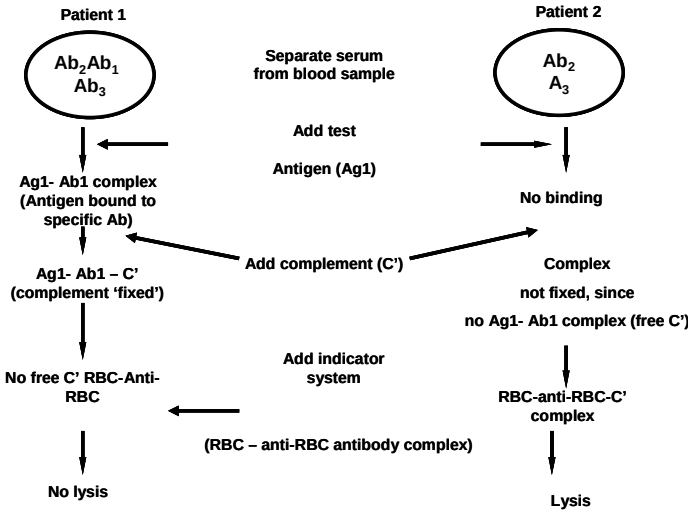


Figure 6.4 complement fixation test

6.2.4 Immunofluorescent Test (IFT)

The fluorescent techniques are extremely specific and sensitive. This technique consists of labeling antibody with fluorescein isothiocyantate, a fluorescent compound with an affinity for proteins to form a complex, conjugate. The

fluorescent assay includes: direct immunofluoresent assay and indirect immunofluoresent assay.

Direct Immunofluorescent assay

In this technique, Fluorescein- conjugated antibody is used to detect antigen- antibody reactions. This method can be applied to the detection of hepatitis B virus & chlamydia. A fluorescent microscope is required to observe the production of color; fluorescein gives a yellow- green light.

Indirect Immunofluorescent Assay (IFA)

This method is based on the fact that antibodies not only react with homologous antigens but can act as antigens and react with antibody.

In the indirect immunofluorescent assay, the antigen source to the specific antibody being tested is fixed to the surface of a microscopic slide. The patent's serum is diluted and placed on the slide to cover the antigen source. If antibody is present in the serum, it will bind to its specific antigen unbound antibody is then removed by washing the slide, finally antihuman globulin conjugated to a fluorescent substance that will fluoresce when exposed to a fluorescent substance that will fluoresce when exposed to ultraviolet light is placed on the slide. This conjugated marker of human antibody will bind to the antibody already bound to the antigen on the slide and will

serve as a marker for the antibody when viewed under a fluorescent microscope.

6.2.5 Enzyme Immuno Assay (EIA)

An enzyme labeled antibody or enzyme labeled antigen conjugate is used in immunologic assays for detection of antigens or antibodies, in a patient's serum e.g. HIV antibody, HIV antigen, hepatitis A antibody.

Various enzymes are employed in enzyme immunoassay. The most commonly used enzymes are peroxidase and alkaline phosphatase. In EIA, a plastic bead or plastic plate is coated with antigen. The antigen reacts with antibody in the patient serum. The bead or plate is then incubated with an enzyme-labeled antibody conjugate, if antibody is present on the bead or plate. The enzyme activity is measured spectrophotometrically after the addition of the specific chromogneic substrate. Test result is calculated by comparing the spectrophotometer reading of patient serum to that of a control serum.

6.2.6 Radio Immunoassay (RIA)

In radioimmunoassay, radioisotopes can be used to measure the concentration of antigen or antibody in serum sample. If

antibody concentration is being measured, radioactive labeled antibody competes with patient unlabeled antibody for binding sites on a known amount of antigen.

The main advantage of the radioimmunoassay method is the extreme sensitivity and ability to detect trace amounts. Of antigen or antibody. In addition, a large number of tests can be performed in a relatively short time period. The disadvantage is the hazards and instability of isotopes.

6.3 Factors Affecting Antigen Antibody Reactions

Many factors affect the interaction between antigen and antibody; these include specificity, cross reactivity, temperature PH, ionic strength, concentration, and intermolecular specificity.

Specificity: The ability of a particular antibody to combine with one antigen instead of another is referred to as specificity. This property resides in the portion of the antigen-binding fragment of an immunoglobulin molecule. Antigen-antibody reactions can show a high level of specificity. Specificity exists when the binging sites of antibodies directed against determinants of one antigen.

Cross reactivity: When some of the determinants of an antigen are shared by similar antigenic determinants on the surface apparently unrelated molecules, a proportion of the antibodies directed against one kind of antigen will also react with the other kind of antigen. This is called cross reactivity. Antibodies directed against a protein in one species may also react in a detectable manner with the homologous protein in another species, which is another example of cross reactivity.

Example of cross reactivity

Three organisms might possess antigenic structures and produce corresponding antibodies as follows:

Organism	Antigens	Antibodies
1	ABC	abc
2	BCD	bcd
3	DE	de

Antiserum prepared from organism 1 will react with organism 1&2 antiserum prepared from organism 2 will react with 1,2, &3 antiserum produced from organism 3 will react organism 2&3 but not with organism1.

Temperature: The optimum temperature needed to reach equilibrium in an antibody-antigen reaction differs for different antibodies. Igm antibodies are cold reacting with thermal

range 4-22^{0}C, and IgG antibodies are warm reacting, with an optimum temperature of reaction at 37^{0}C.

pH: Although the optimum pH for all reactions has not been determined, a pH of 7.0 is used for routine laboratory testing.

Ionic strength: The concentration of salt in the reaction medium has an effect on antibody uptake by the membrane bound erythrocyte antigens. Sodium and chloride ions in solution have inhibition effect. These ions cluster around and partially neutralize the opposite charges on antigen and antibody molecules, which hinders the association of antibody with antigen. Reducing or lowering the ionic strength of a reaction medium such as low-ionic strength salt can enhance antibody uptake.

Concentration: Under normal condition the concentration of antigen and antibody should be optimal but some time this thing fail to be happen in which excess antibody or antigen concentration will result in false reaction, some times known as zonal reaction. When the concentration of antigen is excess it is known as post zone reaction; excess antibody is referred as prozone reaction. This phenomenon can by overcome by serial dilution until optimum amount of antigen and antibody will present.

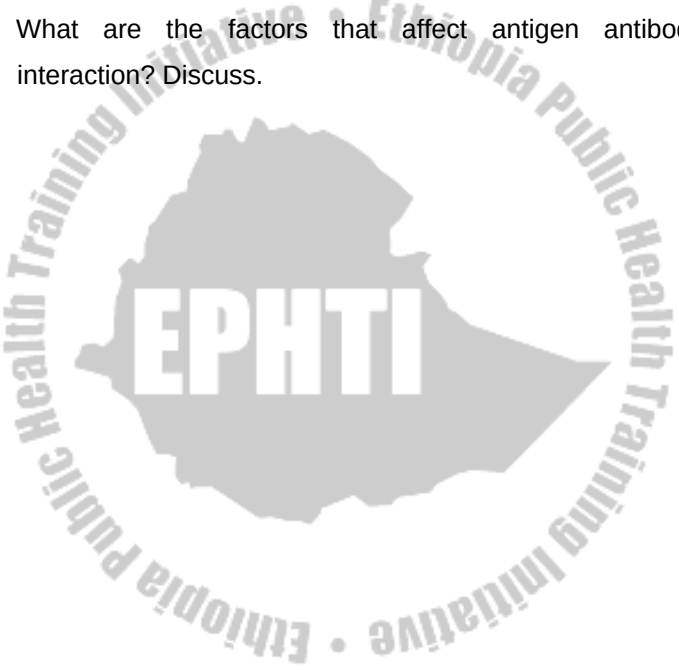
Bond strength and inter molecular attractive force

Bonding of an antigen to an antibody takes place because of the formation of multiple, reversible, intermolecular attraction between an antigen and amino acids of the binding site. The bonding of antigen to antibody is exclusively non covalent. The attractive force of noncovalent bonds is weak when compared to covalent bonds, but the formation of multiple non covalent bonds produces considerable total- binding energy. The strength of a single antigen- antibody bond is termed antibody affinity.

The strongest bonding develops when antigens and antibodies are close to each other and when the shapes of both the antigenic determinate and the antigen-binding site conform to each other. This complementary matching is referred to as goodness of fit.

Review Questions

1. Discuss the difference between single radials diffusion and electro immunoassay
2. Explain the types of agglutination reaction
3. Write the general principle of immunofluoresent assay
4. What are the factors that affect antigen antibody interaction? Discuss.



CHAPTER SEVEN

SEROLOGICAL TECHNIQUES

Learning Objectives

At the end of this chapter, the learner is expected to:

- Know the materials necessary for serological tests
- Practice specimen collection preparation and preservation
- Practice serial dilution & determination of end point ant titer.

7.1 Materials Necessary for Basic Serologic Tests

As discussed in the previous chapter, wide verities of serologic techniques are available to detect either an antibody or antigen. For the detection of this unknown substance from patient's specimen, the specimen should be collected and prepared appropriately. In addition the equipment that is used for testing should be free from any contaminants so as to get true result. The following are some of the equipment used in routine serology.

Glasswares

Dirty glasswares easily affect serological test. After using all the glass wares (test tube, beaker, pipette, etc.) they should be socked in detergent for several hours and rinsed several times in tap water. Finally allow drying by placing in a dry oven or dust free place. Test tubes and pipettes should not be scratched or broken, which will interfere with the reading of a test.

Types of glassware include:

- Test tube
- Glass slides
- Serologic pipette with a size of l0ml, 5ml, 2ml&1ml.

Constant temperature device

Incubator and water bath are usually used in serologic tests. These materials are electrically operated and have thermostat that hold the temperature within the required limits. These devices should be checked prior to use by installing a thermometer

Rotating machines

Rotating machines are required to facilitate antigen antibody reactions. Such machine has a flat plate, which rotate at a prescribed rate of speed. A knob located on the front part of the machine controls the number of revolution per minute.

7.2 Collection Preparation and Preservation of Specimen for Serologic Tests

Specimens that are used for serologic test include: serum, plasma & cerebrospinal fluid. Serum or plasma sample could be obtained from venous blood, which can be performed by the laboratory personnel however. Cerebrospinal fluid should be collected by a physician or a trained nurse.

For serum or plasma sample, first 2-3 ml of venous blood is collected using sterile syringe and needle from a patient. If serum is required, allow the whole blood to clot at room temperature for at least one hour and centrifuge the clotted blood for 10 minutes at 2000 rpm. Then transfer the serum to a labeled tube with a pasture pipette and rubber bulb.

Plasma sample is obtained by treating fresh blood with an anticoagulant, centrifuge and separate the supernatant.

The specimen should be free from hemolyzed blood. Finally, seal the specimen containing tube; the tube should be labeled with full patient's identification (Age, Sex, code n^o, etc). The test should be performed with in hours after sample collection, if this could not be done preserve it at- 20^0c.

7.3 Shipment of Serological Specimen

Most health center and clinic laboratories often are limited in the diagnostic procedures that can be carried out and have to ship serologic specimens to other laboratories. Before shipment the following things should be considered.

- Don't ship whole blood unless the tests to be performed require whole blood.
- Do not inactivate serum or plasma before mailing.
- Keep the specimen and packing container in the refrigerator until time of shipment but if shipment requires several days, freeze the specimen.
- Then ship the specimen by the fastest route.

7.4 Complement Inactivation

Complement inactivation is important because it is known to interfere with different tests. In activation of complement can be achieved by heating the serum or plasma at 56^0C for 30 minutes. If more than four hours has elapsed since inactivation, a specimen should be re-inactivated with same temperature 10 minutes.

7.5 Serial Dilution

Dilution is the act of making a weaker solution from a stronger one. This is usually done by adding a water or saline, which contains none of the material being diluted. Dilution is usually expressed as one unit of the original solution to the total number of units of final solution. Serial dilution means decreasing the volume of serum progressively by maintaining a constant volume of fluid most commonly, serial dilutions are two fold, that is, each dilution is half as concentrated as the preceding one. The total volume in each tube is the same.

Table 7.1. An example of serial dilution

Tube no	1	2	3	4	5
Saline (ml)	1	1	1	1	1
Patient serum (ml)	1	1 of 1: 2	1 of 1: 4	1 of 1: 8	1 of 1: 16
Final dilution	1:2	1:4	1:8	1:16	1:32

7.6 Determination of Endpoint and Titer

If we take the above example again, after serially diluting the patient's serum, equal amount of an antigen is added in each dilution to observe immunolgic reaction. The last tube that shows visible immunologic reaction is known as end point of

the test, the dilution of the antiserum at the end point is known as the titer. The reciprocal of the greatest reacting dilution of the serum is considered as the measure of titer or the concentration of the antibody. For example, it the highest dilution of the serum that shows a visible reaction is at 1:32 dilution, the titer of the test is expressed as 32.

Review Questions

1. Explain how to prepare and preserve a specimen for serologic test
2. Discuss by giving example what serial dilution, endpoint and titer mean.

CHAPTER EIGHT

SYPHILIS SEROLOGY

Learning Objective

At the end of this chapter, the learner should be able to:

- Enumerate the different treponematoses
- Explain the stages of syphilis
- Practice collection of specimen from lesion
- Practice the different tests for syphilis
- Practice preparation of control sera in the laboratory

8.1 Treponematoses

It is a chronic inflammatory disease, primarily it affects the skin and mucous membrane, and during latent period other organs and tissues may be affected. Medically important species is mainly T. palladium but there are other pathogenic treponemes like T. pertenue, T. endemicum & T. carateum.

Each of these organisms are obligate parasite of humans, morphologically identical, cannot be cultured in vitro and have similar laboratory diagnosis and treatment.

These diseases differ in there:

- ✓ Geographical distribution,
- ✓ pathogenicity and
- ✓ degree of virulence.

T. pertenue

Cause a disease known as yaws. Its geographical distribution is West Africa, central Africa south East Asia. T.pertenue is transmitted through exposed skin. Hand, face, legs and feet are parts of the body most affected, it produce raised granular papilloma on the skin. In the later stage, disfigurement of the infected area will be resulted.

T. endemicum

Cause endemic syphilis. It is widely distributed in sub-Sahara Africa and transmitted through exposed skin and oral mucosa.

T.carateum

Cause a disease pinta, has geographical distribution of central and South America, transmitted through exposed skin. The organisms produce itchy red papules on the uncovered part of the body. In the later stage when infected area healed loss of the normal pigment will be resulted.

8.2 Syphilis

Syphilis is caused by a spirochete bacterium, Treponema pallidum.

8.2.1 Morphology and Metabolism of T. Pallidum

Microscopically T. pallidum appears as fine, spiral (8 to 24 coils) organism, approximately 6-15 μm long.

They are not cultivatable with any consistency in artificial laboratory media out side the host. T. pallidum are extremely susceptible to a variety of physical and chemical agents. However, they may remain viable for up to 5 days in tissue specimens removed from diseased animals and from frozen specimens.

Syphilis is a venereal disease. It can be acquired by kissing a person with active oral lesion. There are very few cases of transfusion-acquired syphilis. In addition, syphilis may be transmitted transplacentally to the fetus. Spirochetes can be transmitted to the fetus during the last trimester of pregnancy.

8.2.2 Stages of Syphilis

Untreated syphilis is a chronic disease with sub acute symptomatic periods separated by asymptomatic intervals, during which the diagnosis can be made serologically.

The progression of untreated syphilis is generally divided in to stages. Initially, T.pallidum penetrates intact mucous membranes or enters the body through tiny defects in the epithelium. Upon entrance, the microorganism is carried by the circulatory system to every organ of the body. Spirochetemia occurs very early in infection, even before the first lesions have appeared or blood tests become reactive. Before chemical or serologic manifestations develop patients are said to be 'incubating syphilis". The incubation period usually lasts about 3 weeks but can range from 10-90 days.

Primary syphilis

At the end of the incubation period, a patient develops a characteristics primary inflammatory lesion called a chancre at the point of initial inoculation and multiplication of the spirochetes. The chancre begins as a papule and erodes to form a gradually enlarging ulcer with a clean base and indurate edge. Generally it is relatively painless and commonly located around the genitalia, but in about 10% of

cases lesions may appear almost any where else on the body e.g. Throat, lip, hands.

Most of the patients with primary syphilis will develop swelling of inguinal lymph nods. The primary chancre will persist for 1 to 5 weeks and will heal completely within about 4 to 6 weeks. Primary syphilis is diagnosed by its characteristics chancre with positive serological test and detection of T.pallidum by dark field examination from the lesion.

Secondary syphilis

Within 2 to 8 weeks after the appearance of the primary chancre, a patient may develop the sign and symptoms of secondary syphilis when organisms gain access to the circulation from the infected site.

The secondary stage is characterized by a generalized illness that usually begins with symptoms suggesting a viral infection headache, sore throat, low-grade fever and occasionally nasal discharges. Blood tests reveal a moderate increase in leukocytes with a relative increase in lymphocytes.

The disease progresses with the development of lymhadenopathy and lesions of the skin and mucous membranes corresponding to the spread of the organism in the body by way of the circulating blood. The lesions contain a

large number of spirochetes, and when located on exposed surfaces, are highly contagious. Macular lesions are common, and a rash invariably involves the genitalia and of ten is prominent on the palms and soles.

Secondary syphilis usually resolves it self within 2 to 6 weeks, even in the absence of therapy. It may be diagnosed by typical skin rash and positive syphilis serology test.

Latent syphilis

After resolution of untreated secondary syphilis, the patient enters a latent non-infectious state in which diagnosis can be made only by serologic method. During the first 2-4 years of infection, one fourth of patients will show relapses of manifestation of secondary syphilis. During these relapses, patients are infectious, and the underlying spirochetemia may be passed translucently to the fetus.

Relapses are extremely rare after four years of latency. About one third of patients entering latency are eventually spontaneously cured of the disease, one third will never develop further clinical manifestation of the disease and the remaining one third will eventually develop late syphilis.

Late (Tertiary) syphilis

The first manifestations of late syphilis are usually seen from 3-10 years after primary infection. About 15% of untreated syphilitic individuals eventually develop late benign syphilis characterized by the presence of destructive granulomas.

These granulomas, or gummas, may produce lesions resembling segments of circles that often heal with superficial scarring. Treponems are rarely found in the lesions, which are referred to as benign gummas.

Of untreated patients 10% develop cardiovascular manifestations. T. pallidum may damage large blood vessels such as aorta and coronary arthritis. This condition is usually fatal.

In about 8% of untreated patients, late syphilis involves the CNS. Initially CNS disease is asymptomatic and can be detected only by examination of cerebrospinal fluid. In symptomatic neurosyphilis, spirochetes may also involve the brain tissue and cause destructions of the brain parenchyma (paresis), dorsal root of the spinal cord (tabes).

Congenital syphilis

Congenital syphilis is caused by maternal spirochetemia and transplacental transmission of the microorganism usually after

18 weeks of gestation. Congenital syphilis is diagnosed in three fourths of the cases in patients over 10 years of age. About half of damage to the fetus depends on the stage of the disease and the number of treponemes circulating in pregnant women at the time of transmission.

Early congenital syphilis appears either at birth or up to two years of age, the manifestation includes cutanuous lesion, mucous membrane lesion like thick nasal discharge containing T.pallidum.

Late congenital syphilis may be characterized by fissuring around the mouth, anus, skeletal lesions, and perforation of the palate and the collapse of nasal bones to produce a saddle-nose deformity.

8.2.3 Antibodies in Syphilis

In the treponemes, two classes of antigen have been recognized those restricted to one or a few species and those shared by many different spirochetes. Infection with T.pallidum, T.pertenue, T.carateum T.endemicum produces similar antibody response. Specific and non-specific antibodies are produced in the immunocompetent host.

Specific antibody

Antibodies in early or untreated early latent syphilis are predominantly IgM antibodies. The early immune response to infection is rapidly followed by the appearance of IgG antibodies. The greatest elevation in IgG concentration is seen in secondary syphilis.

Non – specific (nontreponemal or reagin) antibodies

Are produced by infected patients against components of their own or other mammalian cells. Reagin is widespread in nature and can be isolated from any mammalian tissues as well as from treponemes. Although patients with syphilis almost always produce these antibodies, patients with other infectious disease, like measles, hepatitis, leprosy, Brucelosis, malaria, rickettsia, also produce them. Patients can also exhibit reagin with non-infectious conditions such as drug addition, old age, pregnancy and recent immunization.

8.2.4 Collection and Handling of Syphilitic Specimen from Lesion

The treponemal lesion is infectious. As a result wearing a rubber glove is vital to protect one self from infection. The following procedure should be followed to get a representative sample. Cleanse the area of chancre with swab moistened

with physiological saline. Apply gentle pressure on the area to squeeze the sample from the depth of the lesion. Collect the sample of serous exudates on a cover glass and invert it on a slide. Deliver immediately the preparation to the laboratory for examination by dark-field microscopy.

8.3 Tests for Syphilis

Either demonstration of microorganism in a lesion or serologic testing confirms the clinical diagnosis of syphilis in the laboratory. The serologic methods for syphilis measure the presence of two types of antibodies: treponemal and non treponemal.

Serologic procedures for syphilis include the following.

1. Nontreponemal method e.g. Venereal Disease research laboratory (VDRL) and the rapid plasma regain (RPR) procedures.
2. Treponemal methods e.g. Fluorescent Treponema pallidum antibody absorption (FTA-ABS) and microhemagglutination Treponema pallidum MHA-TP).

Nontreponemal methods

The VDRL and RPR are the two most widely used nontreponemal serologic procedures. Each is a flocculation or agglutination test in which soluble antigen particles coalesce

to form larger particles visible as clumps when they are aggregated by antibodies.

The VDRL procedure is recommended when a patient suspected of having syphilis has a negative dark field microscopy result or when atypical lesions are present. It is further recommended that a quantitative VDRL assessment be made quarterly for 1 year after treatment for syphilis, or that the adequacy of treatment in both early and latent syphilis be monitored. The VDRL procedure can be performed on cerebrospinal fluid for the detection of neurosyphilis.

The RPR test can be performed on unheated serum or plasma using a modified VDRL antigen suspension of choline chloride with EDTA. The RPR test card test antigen also contains charcoal for macroscopic reading. It is about as specific as, and possibly more sensitive than, the VDRL slide test.

Treponemal methods

The FTA – ABS and MHA represent treponemal methods. Reactive (Positive) regain test can be confirmed with these two specific treponemal antigen tests. These procedures, however, should not be used as primary screening methods. Procedures such as the FTA-ABS and MHA can be used to confirm that a positive non-treponemal test result has been

caused by syphilis rather than one of the other biologic conditions that can produce positive VDRL, or they can determine quantitative titer of antibody, which is useful in following response to therapy.

The FTA-Abs uses a killed suspension of T. pallidum spirochetes as the antigen.

The micro hemagglutination assay for T. pallidum is based on agglutination by specific antibodies in the patient's serum with sheep erythrocytes sensitized to T. pallidum antigen. The Treponema pallidum immobilization test (TPI) method is obsolete.

Sensitivity of commonly used serologic tests for syphilis

Detection of syphilis by serologic methods is related both to the stage of the disease and to the test method. In the primary stage, about 30% of cases become serologically active after one week and 90% of patients demonstrate reactivity after three weeks. Reagin titers increase rapidly during the first four weeks of infection and then remain stationary for approximately six months. Patients in the secondary stage of syphilis are serologically positive. During latent syphilis there is a gradual return of non-reactive serologic manifestations with non-treponemal method. About one third of patients in the latent stage will remain seroreactive and presumably

infectious. In late syphilis, treponemal tests are generally reactive, non-treponemal methods are non reactive.

Table 8.1 Sensitivity of commonly used serologic tests for syphilis

Test	Stage		
	Primary	Secondary	Late
Non treponemal (regain tests) VDRL RPR	70% 80%	99% 99%	1% (treated late syphilis) 0%
Specific treponemal tests FTA-ABS TPHA-TP	85% 65%	100% 100%	95% 95%
Treponema pallidium Immobilization (TPI)	50%	97%	95%

8.3.1 Venereal Disease Research Laboratory (VDRL) Qualitative Slide Test

Principle: heat inactivated serum is mixed with a buffered saline suspension of cardiolipin–lecithin–cholesterol antigen. This serum-antigen mixture is microscopically examined for flocculation.

Specimen collection and preparation

The specimen should include all identification, it must include the patient's full name, the date the specimen is collected and the patient's hospital identification number.

Blood should be drawn by an aseptic technique. The required specimen is a minimum of 2 ml of clotted blood. The specimen should be promptly centrifuged and an aliquot of the serum removed. Severely lipemic or hemolyzed serum is unsuitable for testing. Before testing, the serum must be heat in activated at $56C^0$ for 30 minutes. In activated serum should be reheated at $56C^0$ for 10 minutes if tested more than 4 hours after the original in activation. Cerebrospinal fluid is also an appropriate fluid for testing.

Reagents required

VDRL antigen – a colorless, alcoholic solution containing 0.03% cardiolipin, 0.9% cholesterol, and sufficient purified

lecithin to produce standard reactivity. Each lot must be serologically standardized by comparison with an antigen of know reactivity. Ampules should be stored in the dark at either at $6C^0$ to 10 C^0or at room temperature antigen that contains precipitate should be discarded.

VDRL- buffered saline

Contains 1% sodium chloride, PH 6.0 $\pm$ 0.1, it should be stored in screw capped or glass stopper bottles

Equipment required

. VDRL test slide with paraffin of ceramic ring.
. 18 gauges hypodermic needle with out bevel (it will deliver 60 drops) ml of reagents.
. 30 ml flat-bottomed glass with stopper, narrow mouth bottle
. Syringe (1-2ml)
. Rotator
. Serological graduated pipette
. Water bath $56C^0$

Preparation of working antigen suspension

1. Dispense 0.4 ml of buffered saline to the bottom of the 30ml, round, glass-stopper bottle.
2. Rapidly add 0.5ml of antigen drop by drop directly by rotating the bottle in a circular motion on a flat surface.

The pipette tip should remain in upper third of the bottle. Take care to avoid splashing saline on the pipette. Blow the last drop of antigen from the pipette without touching the pipette to the saline.

3. Continue to rotate the bottle for 10 seconds.
4. Add 4.1 ml of buffered saline with a 5 ml pipette
5. Place the stopper on the bottle and shake up and down approximately 30 times in 10 seconds. The antigen suspension is ready for use, but it must be gently mixed at the time of use. Do not force back and forth through the needle & syringe as this may lead to break down of antigen particles and loss of their activity.

Note: The working antigen suspension can be stabilized by adding 50 μl of benzoic acid to 5ml of the diluted working solution instead of discarding within 24 hours. The temperature of the buffer saline and antigen should be in the range of 23^0 to $29C^0$. The antigen suspension must be used on the day of preparation.

Quality control

Include positive control sera of graded reactivity each time serologic testing is performed. The antigen suspension to be used each day is first examined with these control sera. Store control sera frozen at -20^0C or liquid form for 7 to 10 days.

Thaw, mix thoroughly, and heat in activate at 56^0C before use.

Check antigen – dispensing needle at the time of use to be sure that it accurately deliver 60-drops/ml reagents. Clean needles and syringes by rinsing with water, alcohol and acetone. Remove needle from syringe after cleaning.

Procedure

1. Pipette 0.5 ml of inactivated patient serum in to one of the rings of the ceramic-ringed slide. Pipette additional specimen and controls in to additional rings.
2. Add one drop of antigen suspension to each serum with a calibrated 18-gauge needle and syringe held in a vertical position.
3. Rotate the slide on a mechanical rotator for 4 minutes. In externally dry climate, cover the slide with a lid containing moistened filter paper to prevent evaporation during rotation.
4. Examine each specimen microscopically with the low (10x) objective.

Note: The test should be performed at a temperature range of 23^0-29^0C.

Reporting Results

Non reactive: No clumping or very slight roughness weakly reactive: Small clumps

Reactive: medium and large clumps

Weakly reactive: small clumps

Note: All reactive and weakly reactive specimens (sera) should be tested quantitatively to estimate the antibody titer. False negative reactions it can occur in a variety of situations like:

- Technical error (e.g. unsatisfactory antigen preparation or techniques.
- The presence of inhibitors in the patient's serum
- Low antibody titer patients may have syphilis, but the reagin concentration is too low to produce a reactive test result. It may be caused by several factors: an infection that is too recent to have produced antibodies, the effect of treatment, latent or inactive disease, or patients who have not produced protective antibodies because of immunological tolerance. These seronegative patients may demonstrate a positive reaction with more sensitive treponemal tests such as the FTA-ABS.
    - Inappropriate temperature
    - Prozone reaction

Weakly reactive results can be caused by

- Very early infection
- Lessening of the activity of the disease after treatment.
- Improper technique or questionable reagents

False – positive reactions can also be observed. Of all positive serologic tests for syphilis, 10% to 30% may be false biologic positive reactions. Non-syphilitic positive VDRL reactions have been reported with cardiolipin type of antigen in rheumatic fever, pneumococcal pneumonia, infectious hepatitis, leprosy, malaria, pregnancy, aging individuals, rheumatoid arthritis. Contaminated or hemolyzed specimens can also produce false positive results.

LIMITATIONS: The VDRL procedure is not specific for syphilis but may demonstrate positive reactions in other reagin-producing disorders, infectious disease and alterations such as pregnancy or aging in normal physiology.

VDRL quantitative test

Principle: Retest quantitatively to an end-point titer all sera that produce reactive, weakly reactive, or questionably non-reactive results in the qualitative VDRL slide test.

Specimen collection and preparation

Same as VDRL Qualitative test for undiluted serum

Preparation of serial dilution.

A. Pipette 0.05 ml of 0.9% saline in to ring number 2,3 &4 on ceramic slide do not spread the saline.

Serum 1-	1	2	3	4
	1:1	1:2	1:4	1:8
Serum 2 -	1	2	3	4
	1:1	1:2	1:4	1:8

B. Pipette 0.05 ml of serum to ring numbers 1 and 2. Draw the serum and saline mixture up and down in the pipette tip in ring number 2 to mix. Aspirate 0.05 ml of diluted serum and spread the remaining dilution over the entire area of the circle with the pipette tip.

C. Transfer 0.05ml of the diluted (1:2) serum in ring number 2 to ring number 3. Draw the serum and saline mixture. Aspirate 0.05 ml of diluted serum and spread the remaining dilution over the entire area of the circle with the pipette tip.

D. Transfer 0.05 ml of the diluted (1:4) serum in ring number 3 to ring number 4 Draw the serum and saline mixture up and down in the pipette tip in ring number 3 to mix. Aspirate 0.05 ml of diluted serum and spread the remaining dilution over the entire area of the circle with the pipette tip.

E. Discard 0.05 ml of the diluted (1:8) serum from ring number 4 unless greater dilutions are needed for strongly relative serum, and spread the remaining dilution over the entire area of the circle with the pipette tip.

Reagent, Supplies, and equipment

In addition to the VDRL qualitative test the following reagent and piece of equipment are needed.

- 0.9% saline

  Preparation – weigh 0.9gm of sodium chloride to a leit volumetric flask. Dilute to the calibration mark with distilled water
- Safety pipette (50ml or 0.05ml)

Procedure:

1. Add one drop of antigen suspension to each diluted serum with a calibrated 18-gauge needle and syringe held in a vertical position.
2. Rotate the slide on a mechanical rotator for 4 minutes.
3. Examine each specimen microscopically (10x objective)

Note: the test should be performed at a temperature range of 23^0 to 29^0C.

Reporting results

Report the titer in terms of the highest dilution that produces a reactive (weakly reactive) result.

Example:

Serum Dilutions				Result
1:1	1:2	1:4	1:8	
Reactive	Reactive	Weakly reactive	Non reactive	Reactive, 1:4 dilution or 4 dilutions

8.3.2 Rapid Plasma Reagin (RPR) Card Test

Principle

A cardiolopin lecithin-cholesterol antigen coated with carbon particle is mixed with patient's serum. Then flocculation reaction is observed macroscopically in the presence of cardiolipin antigen.

Specimen collection and preparation

No special preparation is required before specimen collection. The specimen should be labeled with all patient's identification fresh serum or plasma sample can be used. It is not important to heat inactivate the specimen before testing.

Reagents & Equipment

Note: Except for the antigen, all other components should be stored at room temperature in a dry place in the original kit packaging.

Provided in the test kit

RPR card test antigen

It contains cardiolipin, lecithin, cholesterol, EDTA, charcoal, chorine chloride, and distilled water. Store the antigen suspension in ampules or in plastic dispensing bottle at 2^0 to 8^0C. Unopened ampules have a shelf life of 12 months from the date of manufacture.

Opened antigen ampules has stability for 3 months

- Needle, 18-gauge, without bevel. The needle should deliver 60$\pm$2 drops of antigen suspension per milliliter when held in a vertical position.
- Specially prepared, plastic-coated cards
- Serological pipette
- Dispenser, 0.05 ml/drop
- Stirrer

Other material

- Rotator
- Humidifier cover containing a moistened sponge
- 0.9% saline

Quality control

A. Controls with established patterns of graded reactivity should be included in each day's testing to confirm optimal reactivity of the antigen suspension. Control sera must be at 23^0 to 29^0C at the time of testing.

B. Calibrate the delivery needle before testing

Procedure

1. Place 0.05ml of unheated serum on the test card with a serologic pipette. Don't touch card surface.
2. Spread serum in the circle with stirrer
3. Gently shake antigen-dispensing bottle before use. Hold the needle in a vertical position; dispense exactly one free-falling drop (1/60 ml). Do not stir. Mixing is accomplished during rotation.
4. Place card on rotator and cover with humidifier cover. The card can be hand rocked and used where laboratory equipment is not available.
5. Rotate 8 minutes at 100 rpm on mechanical rotator.
6. Observe the specimen immediately

Reporting results

Reactive: slight to large agglutination (black clumps)

Non-reactive: no agglutination, or very slight roughness (even light – gray color).

Sources of error

- Error can be introduced in to test results because of factor such as contamination of rubber bulbs or improperly prepared antigen suspension.
- Biological false positive occur in the following conditions like rheumatic fever, viral pneumonia, hepatitis, leprosy, malaria, pregnancy, aging individuals.
- False negative reaction can result from poor technique, in ineffective reagents or improper rotation.
- If mechanical rotation is below or above the 95 to 110 rpm (acceptable range) there is a tendency for the clumping of antigen to be less intense in procedures with undiluted specimen, so that some minimal reactions may be missed.

8.3.3 Fluorescent Treponema Pallidum Antibody Absorption Test

Principle: Patients serum is added on the slide coated with T. pallidum antigen followed by addition of fluorescent-tagged anti-human globulin and examine under fluorescent microscope. It's the most sensitive confirmatory test

Specimen collection and preparation

The serum should be heat inactivated

The specimen should be labeled with all the identification

Reagent supplied

Treponema pallidum antigen: Extracted from rabbit testicular tissue

Store at 6^0_c -10^0C in lyophilized form

Conjugate (fluorescent labeled anti-human globulin)

Positive control

Equipment

. Test tubes, pipette

. Incubator

. Fluorescent microscope

Procedure

1. Coat the slide with T.pallidum antigen by adding a drop of suspension of T.pallidum on a clean slide and keep in oven at low temperature
2. Take out and wash by rinsing with tap water to remove excess unbounded T.pallidum
3. Add the patient's serum to the coated antigen and incubate, rinse by tap water to remove excess antibody, if the pt serum has an antibody, and to remove the whole serum if it doesn't contain antibody.
4. Add conjugate (fluorescent tagged antihuman globulin) and rinse with tap water, to remove excess conjugate, if

serum contain antibody and to remove the whole conjugate, if it doesn't contain antibody.

5. Examine under fluorescent microscope.

   The fluorochromes usually used are

   - Fluorescein isothyocyanate – yellow green
   - Rudamin - Red color

Reporting results

Fluorescence indicate the presence of specific antibody to T.pallidum

Non – fluorescence indicate the absence of specific antibody to T.pallidum.

8.3.4 Preparation of Control Sera

1. Collect all reactive sera and store in freezer
2. Collect all non reactive sera and store in freezer
3. After collecting a large amount thaw at room temperature
4. Filter to remove the particles
5. Add mertiolet (preservative) 1 mg/ml serum.
6. Make a serial dilution of reactive with non reactive sera
7. Perform several tests each day. Select the dilution, which always gives reactive, weakly reactive and non-reactive result.
8. Prepare a large quantities of those dilution

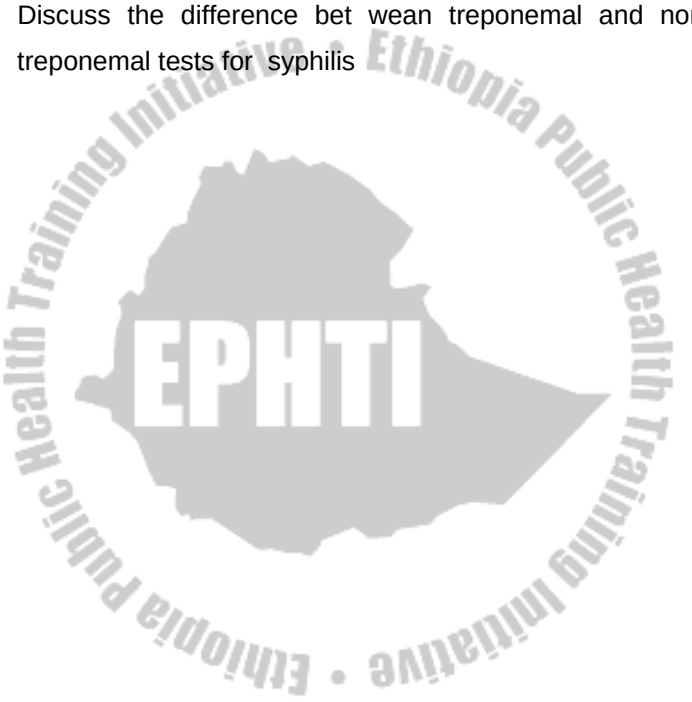
9. Distribute in a small container (alginate) and store in a freezer.
10. Control sera of graded reactivity should be included each time when serologic procedures are performed.

NB: If the control sera fail to give the desired (known) result (do not produce the established relativity) pattern the result of the specimen is unacceptable so to have acceptable result the following should be done:

- Prepare another antigen suspension
- Test temperature must be adjusted at room temperature (23-29^{0}C)
- Adjust equipment
- Use commercially prepared controls
- Strictly follow the manufactures procedure.

Review Questions

1. Discuss the different stages of syphilis
2. Explain how specimen is collected from lesion of syphilitic patients
3. Discuss the difference bet wean treponemal and non treponemal tests for syphilis



CHAPTER NINE

AGGLUTINATION TEST FOR FEBRILE DISEASE

Learning Objective

At the end of this chapter, the learner should be able to:

- Explain the etiology and way of transmission of febrile disease
- Practice widal and Weil-felix tests

When any pathogenic microorganism invades the human body, the natural response is the production of antibodies. The host and microbial factors influence the rate of antibody formation, the type and amount of antibodies produced, and the persistence of antibody in the circulation. Among the antibodies produced in response to certain pathogenic microorganism are febrile agglutinins. The microorganism that elicits the production of febrile agglutinin is characterized by presence of persistent fever & frequently difficult to grow in laboratory cultures. Some of the causative agents of febrile diseases are salmonella species, rickettsial and brucella abortus.

9.1 Typhoid and Paratyphoid Fever

The etiological agent is Salmonella species; it occurs in human only. Some times it is termed as enteric fever since they colonize the intestine. Salmonella of medically important species are S.typhi (typhoid fever), S.paratyphi A and B (paratyphoid fever).

Typhoid and paratyphoid fever is transmitted through ingestion of contaminated food or water. They contaminate usually by carriers like rodents, hens, cows, etc. Typhoid or enteric fever is a clinical syndrome characterized by fever, headache, splenomegaly, leucopenia & cough.

Its incubation period ranges from 7 to 14 days. In 5% to10% of untreated patients relapse may occur the symptoms in relapse are milder than the initial illness and begin about two weeks after discontinuation of antimicrobial therapy. The carrier state is assymptomatic and in 1 - 3% of carriers there is continuous excretion of S.typhi for a minimum of one year. The gall bladder is the site of persistent intestinal infection.

Identification of salmonella

Salmonella species can be identified based on their antigenic structure they possess. They have three different antigenic structures.

O- antigen (somatic antigen)

It is lipopolysaccharide of the outer membrane, which is heat and alcohol stable antigen. Salmonella is divided in to five distinct serogroups (A-E) on the basis of somatic antigen.

H-antigen (flagellar antigen)

H-antigen is protein, which makes the perithrchous flagella. It is heat and alcohol labile. Salmonella is further subdivided in to more than 1200 serotypes on the basis of flagellar antigens.

Vi- Antigen:

This is the antigen that determines the virulence, the ability to cause disease, of the organism.

Preparation of antigen suspension

Salmonella antigen suspension is available commercially and it's also possible to prepare in the laboratory.

A. Preparation of H antigen (flagellar antigen)

Procedure

1. Inoculate bacteria from a single colony in to a broth and incubate for 6hrs.
2. View a drop of the culture in a wet film to confirm that most of the bacteria are motile and therefore sufficiently flagellated for the tests.

3. Kill the culture by adding formaldehyde to a concentration of 0.2% and incubate for several hours at 37^0C

B. Preparation of O antigen

Procedure

1. Suspend the bacteria from an agar culture in saline and heat for 30 minute at 100^0C to remove the flagella.
2. Centrifuge to separate the bacteria from the detached flagella.
3. Resuspend the bacteria in saline.

   Alternatively

   1. Remove the flagella by mixing a dense saline suspension of the bacteria with an equal volume of absolute ethanol
   2. Incubate for 20 hr at 37^0C
   3. Dilute the suspension with saline.

Widal test

Widal test is a serological test, which is commonly used to diagnose typhoid and paratyphoid fever. The patient's serum is tested for O and H antibodies

Rapid slide (Screening) test

1. Clean the glass slides supplied in the kit well and wipe it free of water.

2. Place one drop of undiluted test serum in each of the first circle (1to4) and one drop of positive control serum in each of the last two circles.
3. Place one drop of antigen O, H, A (H) and B (H) in circle 1,2,3, &4 respectively and O antigen in circle five and H antigen in circle 6
4. Mix the contents of each circle with separate applicator stick and spread to fill the whole area of the individual circle.
5. Rotate the slide for one minute and observe for agglutination.

If agglutination is visible, quantitative estimation of the titer of the appropriate antibodies should be done

Tube agglutination method

Procedure

1. Take a set of 8 clean dry test tubes for each serum to be tested.
2. Place 1.9ml of saline in tube 1 and 1 ml of saline in other tuber (2-8)
3. Transfer 0.1 ml of undiluted serum to tube 1. Mix thoroughly. The resultant dilution of serum is 1:20.
4. Further dilutions are done in the following
   a) Transfer 1ml of the diluted serum from tube 1 and place in tube 2 this leads to 1:40 dilutions in tube2
   b) Repeat the transfer process for tube 7 after mixing.

c) Leave 1 ml of saline in tube 8 at the 'saline control'

Note. Tube 1 has a serum dilution of 1:20, 1:40 (2), 1:80 (3), and 1:160 (4), 1:320 (5), 1:640 (6) & 1:1280 (7).

5. Add one drop of appropriate antigen in each (use only that antigen suspension which has given a positive reaction in the screening test).

   Note: Each antigen (O, H, AH, BH) will require a series of 8 tubes for determine the titer of their corresponding antibodies.
6. Mix well and incubate overnight (16-18 hrs) at 37^0C.
7. Examine agglutination macroscopically.
8. Two tubes for positive control O and it antigen should be included.

Interpretation

1. Only a titer above 1:80 should be considered as significant.
2. A rise in titer (done each week) is considered to be definite evidence of infection. A single test result is considered of diagnostic value only when it is usually high (above 160).
3. Antibiotic treatment in typhoid fever often prevents a rise in titer,
4. A negative test does not rule out the possibility of infection because of the tine when the blood sample was taken in relation to the stage of the disease.

5. Positive results should always be interpreted with reference to clinical findings.

9.2 Rickettsial Disease

Rickettsiae resemble viruses in that they are obligate intercellular parasite and unable to survive as free-living organisms. They are about the size of the large viruses and can just be seen with the light microscope. Unlike viruses rickettsiae contain both RNA and DNA multiply by binary fission, they have cell wall that contains muramic acid and enzyme.

Based on their antigenic structure, the genus rickettsia has been divided into three main groups: typhus group (R. prowazeki, R. typhi), scrub typhus group (R. tsutsugamushi), spotted fever group (R.conori, R.siberica, R.rickettsi R.conoripijperi).

Man is an accidental host of rickettsia species except R. prowazeki; they live in intestinal tract of louse, fleas, ticks and mites. Reservoirs host include, dogs, rats, mice, rodents Rickettsial disease can be acquired by inhaling of dried infected vector faces, through damaged skin bite of an infected vector ticks, mite, etc. The infection is characterized by high continuous fever, severe head ach and body pains,

marked weakness, enlarged spleen

Laboratory diagnosis

Embryonated egg inoculation technique used for culturing viruses can also be used for isolating rickettsiae however it require costly materials and performed in reference laboratory.

Serology

In rickettsial infection, specific IgM antibodies are produced followed by IgG response in the later stages. The most reliable and useful serological techniques to diagnose rickettsial infections are immunofluoresent assay and complement fixation test; however, this test is not available in district laboratory due to its cost

Weil-felix reaction

A Weil Felix test is a type of agglutination test most commonly used serologic test. The reaction is based on similarity of particular antigenic determinant, which occur in most species of pathogenic rickettsia and in the OX-19, OX -2 strains of Proteus vulgaris and OX-K strains of Proteus mirabilis. In other word, Proteus antigen is used to detect rickettsial antibody. This could be an example of hetrophile antigen antibody reaction. Weil Felix test has similar principle and procedure with Widal test.

Table 9.1 Weil-felix reactions in Rickettsial infection

Weil-Felix Reaction			
Organisms	OX-19	OX-2	OX-K
TYPHUS GROUP			
R.prowazeki	+++	+/-	-
R.typhi	+++	+/-	-
SCRUB TYPHUS TROUP			
R.tsutsugamclshi	-	-	+++/-
SPOTTED FEVER GROUP			
R. conori	+/++	+/++	-
R.conoripijperi	+/++	+/++	-
R.siberica	+/++	+/++	-
R.rickettsi	+/++	+/++	-

+++ frequent and large antibody response

- no antibody response

Note: False negative reactions are common in scrub typhus. False positive reactions may occur in Proteus infections, relapsing fever, brucellosis and other acute febrile illnesses.

A rise in titer in two consecutive specimen collected in interval is significant than a rise in titer in single specimen and a rise in titer in single specimen should not be taken as a positive sample.

9.3 Brucella Abortus

Brucella aborts, gram-negative bacilli, is the causative microorganism of brucellosis. If is a zoonoses that infects humans by accident. The agents of brucellosis are normal flora of the genital and urinary tract of many animals including pigs, cows, and dogs. Most human acquire brucellosis because of the ingestion of contaminated food products or through occupational exposure. Farmers, veterinarians, and slaughterhouse workers are particularly prone to infection.

Laboratory diagnosis

Because of the difficulty of isolating this organism by the culturing technique, many cases of brucellosis are diagnosed serologically by identifying the presence of antibodies. Antibodies usually appear within 2 to 3 weeks after infection. An antibody titer of 1:80 to 1:60 strongly suggests infection.

Review Questions

Say true or false

1. Typhoid and paratyphoid fever can be diagnosed by Widal test
2. In weil-felix test, some strains of Proteus vulugaris and proteus mirabilis antigen is utilized as reagent
3. Brucella abortus commonly affect human being.

CHAPTER TEN

HUMAN CHORIONIC GONADOTROPIN HORMON (HCG)

Learning Objective

At the end of this chapter, the learner will be able to:

- Practice specimen collection for pregnancy test
- Practice the different tests for pregnancy
- Explain factors that affect pregnancy tests

10.1 HCG and Pregnancy

Human chorionic gonadotropin (HCG) is a hormone secreted by placenta during pregnancy. Its production stimulates secretion of progesterone by the ovary.

Adequate levels of progesterone are necessary for successful implantation and prevent any further release of egg from the ovary.

Human chorionic gonadotrophin is a glycoprotein, has alpha and beta sub units. The alpha subunit usually cross react with

the alpha subunit of leutenizing hormone, however, the beta subunit is specific for HCG. It appears in urine, blood and amniotic fluid. The serum and urine level rise rapidly during gestation, reaching a peak at six to eight weeks, after which there is a steady decline.

10.2 Pregnancy Tests

Laboratory tests for pregnancy are based on the detection of human chorionic gonadotrophin hormone in serum or urine; mainly there are two types of test.

I. Biologic animal Bioassay (A-Z test)

This test is performed in laboratory animal (female mouse). i.e. Patient's urine is injected in to a female mouse after certain period, the mouse will be killed and the ovary will be examined for sign of pregnancy.

However, this test cannot be used for early diagnosis. Moreover it is time consuming and requires steady supply of laboratory animals.

II. Immunologic test

Immunologic test could be qualitative and quantitative. Qualitative estimation of HCG in urine is used for early detection and confirmation of pregnancy. Quantitative

estimation of HCG in serum has of value in case of pre-eclamptic toxemia, hydatidiform mole and choriocarcinoma. Compared to biologic animal assay, immunologic test is less expensive and quicker test.

10.3 Specimen Collection

An early morning urine specimen is preferable because this is the most concentrated and contains the highest level of HCG. However, specimen collected at any time may be used with a specific gravity at least 1.010. Urine must be collected in a clean detergent free container. If it cannot be tested immediately, it should be refrigerated at 4^0C for not longer than 48 hours. Specimen preserved with boric acid is also suitable for testing.

When tested, the urine and test reagents should be at room temperature. If the urine is cloudy it should be filtered or centrifuged and the supernatant fluid used.

Specimens that are heavily contaminated or contains large amount of proteins or blood, are not usually suitable for testing.

There are two types of immunologic test commonly available and provided in a form of kit.

- Rapid latex slide test: have two types
  I. Indirect latex slide test
  II. Direct latex slide test
- Tube test (haemagglutination inhibition technique)

A. Rapid latex slide test

I. Indirect latex slide test

Principle

Urine specimen is first treated with anti-HCG and then reacted with the latex suspension. If the urine contains HCG, the anti HCG will be neutralized and then the latter will not be available to the HCG coated latex particles for bringing about agglutination.

Reagents and materials

. Antiserum that contain HCG antibody
. Latex reagent coated with HCG
. Positive and negative controls
. Mixing sticks and slides

Procedure

Note: In all case it is better to refer manufacturer manual

1. Place one drop of urine sample on the ring of the slide provided by the manufacture.

2. Add one drop of anti-HCG reagent to the urine specimen placed on the slide. Mix the two fluids well with applicator stick.

   Note. In order to maintain the same volume, always hold the dropper in the same vertical position and use the same vertical position and use the same kind of dropper for both urine specimen and the antiserum.
3. Rock the slide gently for about 30 seconds
4. Gently shake the vial with latex antigen and then add one drop.
5. Mix again with applicator stick and observe the appearance of agglutination at 2 minutes under a bright light.

Reporting

Latex particle agglutinated _____ Negative (non-reactive)
Homogenous suspension of latex particles with out any sign of agglutination _____ Positive (Reactive)

II. Direct latex slide test

Principle

The reaction is based on the reaction between HCG in urine and the latex particles coated with anti HCG. In positive result agglutination will be observed.

B. Haemagglutination inhibition test (tube test)

It is more sensitive than slide test

Principle: similar with latex slide test except the HCG is coated on red cells, not on polystyrene particles.

Procedure

1. Add a drop of urine and drop of anti-HCG antiserum in a small tube.
2. Add red cell coated with HCG
3. Mix the contents of the tube and leave at room temperature (20-28^{0}C) for 1-2 hrs to allow time for the red cells to settle.
4. If the urine contains HCG it will combine with the antibody. This will leave no antibody to react with the HCG on the red cells.
5. If the urine contains no HCG, the anti HCG antibody will react with the HCG on the red cells and cause their agglutination.

Reporting

Reactive _____Non-agglutinated cells settle in the bottom of the tube.

Non-reactive _____Agglutinated cells settle and covers the bottom of the tube.

10.4 Factors Affecting Pregnancy Tests

Different factors influence the result of pregnancy test these are

False Negative may occur in conditions like:

- Error in reading – inappropriate interpretation of procedure
- Test is performed too early-The concentration of HCG is below the sensitivity of the test, which is capable of detecting reliably. The sensitivity of a test, the recommended time of testing will be included in the information supplied by the manufacture.
- Urine too diluted -falsely low levels of HCG may be due to a diluted urine (low specific gravity)
- Ectopic pregnancy implantation of the ovum outside the uterine cavity

False positive may occur in conditions like

- Error in reading- inappropriate interpretation of test procedure
- Luteinizing hormone cross-reaction
- Test performed at time of ovulation or in menopausal women
- Proteinuria and hematuria
- Recent pregnancy -test performed less than 10 days after abortion of full-term delivery.

- Detergents on glassware and slide used in the test, it must be well rinsed to remove trace of detergent even the smallest trace of detergents may affect the performance of the test.
- Drug interference- aldomet, marijuana, aspirin in large doses, etc.
- HCG treatment for infertility
- Trophoblastic disease e.g. molar pregnancy or choriocarcinoma
- HCG secreted by malignant tumor (ovary, breast, lung, kidney)
- Testicular tumor (in male)

Use of pregnancy test

Situations in which pregnancy testing is indicated include pregnancy test usually ordered to investigate some conditions like ectopic pregnancy, threatened abortion, hydatiform mole, and choriocarcinoma. It also used for checking a woman of childbearing age is pregnant before carrying out medical or surgical investigation, x-ray or drug therapy that could be harmful to an embryo.

Review Questions

1. Explain the difference between direct and indirect latex slide test.
2. Discuss the commonest causes of errors in pregnancy test.

CHAPTER ELEVEN

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Learning Objective

At the end of this chapter, students are expected to:

- Explain the disease characteristics and clinical manifestation of HIV.
- Describe and practice the laboratory diagnosis of HIV.

HIV is a member of the family retroviridae, a type D retrovirus that belongs to the lentivirus subfamily. HIV-1 and HIV-2 are medically important viruses. The two viruses are 40% similar in their overall structures, and both can cause AIDS. HIV-1 is responsible for the main AIDS epidemic and common in Ethiopia.

The virus transmits through sexual intercourse, blood transfusion or by contaminated needle. Sexual transmission, either heterosexual or male-to-male is a well-documented route of transmission. Children born to women with HIV have a 20% to 30% risk of HIV infection. Infected mothers can also spread HIV to their newborn infants by breast-feeding.

11.1 Disease Characteristics and Clinical Manifestation

Infection with HIV produces a chronic infection with symptoms that range from a symptomatic to the end stage complications of AIDS.

Typically, patients in the early stages of HIV infection are either completely asymptomatic or may show mild, chronic swelling of lymph nodes. The early phase may last from many months to many years after viral exposure.

During the early period after primary infection, widespread dissemination of virus occurs and a sharp decrease in the number of CD_4 T- Cells in peripheral blood is manifested. The early burst of virus in the blood is often accompanied by flu-like symptom.

This phase is followed by a prolonged period of clinical latency range 7 to 11 years. During this period the patient is usually a symptomatic. Due to different factors, there is a variation in the duration of clinical latency.

The quantity of CD_4 lymphocytes continues to diminish as the disease progresses and when the number of cells reaches a critically low level the risk of opportunistic infection increases.

Clinical symptoms of the later phase of the disease include extreme weight loss, fever and multiple secondary infections. The end stage of AIDS is characterized by the occurrence of opportunistic infection like M. tuberculosis, Salmonella, P. carinii, etc.

11.2 Laboratory Diagnosis

A 'window' period of seronegativity exists from the time of initial infection to 6 or 12 weeks or longer.

Serological screening tests designed to detect HIV antibodies are usually enzyme linked immunosorbent assay and dot blot assay; western blot assay is commonly used confirmatory test.

Enzyme Linked ImmunoSorbent Assay (ELISA)

The indirect ELISA is the most commonly utilized test that is supplied in the form of kit. In this type of assay, an antigen coated on a solid phase combine with the patient's serum containing antibody, the antigen antibody complex will interact with conjugate (enzyme Labeled with anti human immunoglobulin) then a color change is observed up on addition of a substrate. The intensity of the color gives an indication of the amount of bound antibody.

Dot blot assay (HIV spot test)

Dot blot assay are rapid and easy to perform. In this type of assay antigens are coated on micro particles that are trapped within a membrane. These antigen bind with HIV antibody on the patient's sample than a color production is observed when the conjugate is added on antigen antibody complex.

Western blot assay

Western blot assay is the most widely accepted confirmatory assay for the detection of HIV; however, it is time consuming and expensive test. In the western blot procedure, purified HIV-1 viral antigens are electrophoresed on sulfate polyacryamide gel (SDS gels) and the separated polypeptides are then transferred on to sheets of nitrocellulose paper incubated with the serum specimen. Any antibody that binds to the separated peptides present on the nitrocellulose paper is detected by a secondary antihuman antibody, conjugated to enzyme substrate. Antibody specificities against known viral components are considered true positive results, whereas antibodies specific against nonviral cellular contaminants are nonspecific, false-positive results.

Review Questions

1. Explain what ‘window’ period mean in HIV infection
2. Discuss the screening tests for HIV diagnosis

CHAPTER TWELVE

HEPATITIS

Learning Objective

At the end of this chapter, students should be able to:

- Explain the different types of viral hepatitis
- Discuss the different laboratory tests for viral hepatitis.

Viral hepatitis is the most common liver disease worldwide. The viral agents of acute hepatitis can be divided in to two major groups

1. 1^0 hepatitis viruses: A, B, C, D & E
2. 2^0 hepatitis viruses: Epstein- Barr virus, cytomegalovirus, herpes virus, etc.

Primary hepatitis viruses attack primarily the liver and have little direct effect on other organ system. Secondary viruses involve the liver secondary in the curse of systemic infection of another body system.

Hepatitis A virus

(Infectious or short- incubation hepatitis)

Hepatitis A virus is a small, single stranded RNA virus when seen by electron microscope. Infection can be acquired by

ingestion of virus in contaminated food or water from hands or other objects contaminated with infected feces (fecooral routes), after exposure within 2-6 weeks clinical symptoms will develop.

In acute phase of infection, the highest titers of HAV can be detected in stool sample.

Shortly after the onset of fecal shedding, an IgM antibody is detectable in serum, followed with in a few days by the appearance of an IgG antibody. IgM anti-HA is almost always detectable in patients with acute HA. IgG anti-HA, a manifestation of immunity, peaks after the acute illness and remains detectable indefinitely, perhaps lifelong. The finding of IgM anti- HA in a patient with acute viral hepatitis is highly diagnostic of acute HA. Demonstration of IgG anti-HA indicates previous infection. The presence of IgG anti-HA protects against subsequent infection with HA virus, but it is not protective against HBV or other viruses.

Laboratory diagnosis

Testing methods for hepatitis A virus include the following:

1. Total antibody by enzyme immunoassay (EIA)
2. IgM antibody by RIA
3. HA antigen by radioimmunoassayt (RIA)

Hepatitis B virus

(Long term or serum hepatitis)

Hepatitis B virus is a double stranded, DNA virus. It is the classic example of a virus acquired through blood transfusion. It has various antigens hepatitis B surface antigen, an outer coat, hepatitis B antigen that is an inner core component and hepatitis B core antigen.

The major routes of transmission of hepatitis B virus included blood transfusion, sexual inter course, transplacental and sharing of contaminated needle. The incubation period of hepatitis B virus may range from 6-26 weeks.

Infected patients manifest hepatitis B virus in all body fluids including blood, feces, urine, saliva, semen, tear and milk.

Laboratory diagnosis

Serum that is collected in acute stage of illness can be tested by:

- Counterimmunoelectrophoresis
- Enzyme Linked immuno sorbent assay
- Reverse passive Hemagglutination test

Reverse passive hemagglutination is the commonly employed test since it is less expensive and sensitive test.

Hepatitis C virus

Viral hepatitis caused by hepatitis c virus, the identification of this virus is not clear, sometime it known as non-A/non-B hepatitis. This virus is commonly acquired by contaminated blood and blood products.

Laboratory diagnosis

Hepatitis C virus antibody can be detected from serum usually by radioimmunoassay.

Hepatitis D virus (Delta virus)

It is defective or incomplete RNA virus that is unable by itself to cause infection, i.e., transmitted through blood products.

HBV is required as a helper to initiate delta infection only persons with acute or chronic HBV infection can be infected with delta agent.

Laboratory diagnosis- Radio immuno assay

Hepatitis E virus

This is responsible for large water borne out breaks incubation period 6 weeks.

Laboratory diagnosis: Specific test for IgM & IgG anti-hepatitis E virus.

CHAPTER THIRTEEN

C-REACTIVE PROTEIN

Learning Objective

Upon completion of this chapter, students are expected to:

- Describe clinical significance of CRP and other plasma proteins
- Practice the test used for detection of creative proteins

13.0 C-Reactive Protein

The main biologic sign of inflammation is an increase in the erythrocyte sedimentation rate (ESR). In addition an increase in plasma concentrations of a group of proteins known as acute-phase proteins is a good indicator of local inflammatory activities and tissue damage. The acute phase proteins include C-reactive protein (CRP), inflammatory mediators (e.g. complement components c_3 and c_4, fibrinogen, etc.

CRP is prominent among the acute-phase proteins because it provides fast and adequate information of the actual clinical situation; as a result CRP is a direct and quantitative measure of the acute-phase reactions.

Measures of CRP add to the diagnostic procedure in selected cases (e-g. in the differentiation between a bacterial and a viral infection). An extremely elevated CRP is suggestive of a possible bacterial infection. The CRP level may be useful also for monitoring the effect of treatment and for early detection of postoperative complications or intercurrent infections. The CRP is a parameter for inflammatory activity.

CRP is a method of choice for screening for inflammatory and malignant diseases and monitoring therapy in inflammatory disease. Elevations of CRP occur in nearly to diseases states, including bacterial infection, viral infections, myocardial infraction specificity rules out CRP as a definitive diagnostic tool.

The CRP test has been widely used to detect infection in circumstances where microbial diagnosis is difficult. These conditions include septicemia and meningitis in neonates, infections in immunosuppressed patients, serious post operative infections etc.

CRP levels rise following the tissue injury or surgery. In uncomplicated cases the level of CRP peaks about 2 days postoperatively and gradually returns to normal levels within 7 to 10 days. CRP is synthesized more rapidly than other acute

phase proteins; assays of CRP are the measurement of choice in suspected inflammatory conditions.

Tests for CRP

Rapid latex agglutination test

Principle: The test is based on the reaction between patient serum containing CRP as the antigen & the corresponding antibody coated to the treated surface of latex particle. The coated particles enhance the detection of an agglutinate reaction when antigen is present in the serum being tested.

Specimen- Serum

Reagent & materials required

- CRP latex reagent
- Glycine – saline buffer
- Capillary pipette
- Applicator sticks
- Glass slide
- Serologic pipettes & rubber bulb

Quality control

Include positive & negative control serum.

Procedure

1. Deliver one drop of undiluted serum on a slide by using capillary Pipette.

2. Deliver one drop of positive and negative control on separate (other) circle of the slide
3. Add one drop of CRP latex reagent to each serum specimen & to each control
4. Mix the suspension using separate applicator sticks.
5. Tilt the slide back & forth slowly for two minutes observe for agglutination macroscopically.

Reporting

Positive reaction – agglutination

Negative reaction– absence of agglutination

Review Questions

1. Discuss how CRP produced in the body

CHAPTER FOURTEEN

INFECTIOUS MONONUCLEOSIS

Learning Objectives

At the end of this chapter, students should be able to:

- Describe the etiology of infectious mononucleosis
- Explain the hetrophile antibodies
- Discuss the serologic test

14.1 Epstein – Barr Virus

The Epstein – Barr virus (EBV) is the cause of infectious mononucleosis. It is an important factor in the development of nasopharyngeal carcinoma, an epithelial cancer.

EBV infection can result in complications involving the cardiac, respiratory, digestive & renal system.

EBV is a human herpes DNA virus. In infectious mononucleosis the virus infects B-lymphocytes

Although EBV appears to be transmitted primarily by close contact with infectious oral-pharyngeal secretions, the virus

has been reported to be transmitted by blood transfusion and transplacental.

The incubation period of infectious mononucleosis is from 10 to 50 days. Clinical manifestations include sore throat, fever, and fatigue.

14.2 Hetrophil Antibodies

Hetrophil antibodies comprise a broad class of antibody. These antibodies are defined as ones that are stimulated by one antigen react with an entirely unrelated surface antigen present on cells from different mammalian species.

Hetrophile antibodies may be present in normal individuals in low concentrations but a titer of 1:56 or greater is clinically significant in suspected cases of infectious mononucleosis.

The IgM type of hetrophil antibody usually appears during the acute phase of infectious mononucleosis, but the antigen that stimulates its production remains unknown. The following features characterize IgM hetrophil antibody:

- Reacts with horse, ox and sheep erythrocytes
- Absorbed by beef erythrocytes
- Not absorbed by guinea pig kidney cells
- Does not react with EBV-specific antigens.

14.3 Serologic Tests

Paul and Bunnell first associated infectious mononucleosis with sheep cell agglutination and developed a test for the infection mononucleosis hydrophilic. Davidson modified the original Paul Bunnell test, introducing a differential adsorption aspect to remove the cross-reacting. Forssman and serum sickness hetrophil antibodies. Since then the Davidson test has become the classic laboratory reference test for the diagnosis of infectious mononucleosis. However, this test is time consuming and cumbersome.

Rapid slide tests, based on the principle of agglutination of horse erythrocytes appears to increase the sensitivity of the test

Review Questions

1. Explain the characteristics of hetrophil antibodies
2. Discuss the serologic tests for hetrophil antibodies

CHAPTER FIFTEEN

STREPTOLYSIN O

Learning Objective

At the end of this chapter, students are expected to:

- Explain the properties streptolysin O and streptolysin S.
- Practice the tests for streptolysin O.

15.0 Streptolysin O

Streptolysin O is a hemolytic factor produced by most strains of GroupA beta- hemolytic streptococci (S. pyogenes). Streptococci are gram-positive cocci in chain, non-motile, facultative anaerobes. It produce toxin like streptolysin O & streptolysin S and enzymes like DNAase, streptokinase. It is oxygen & heat labile immunogenic enzyme with molecular weight range from 50,000-75,000 Dalton, which cause lysis of red cells under reduced condition. It can severely damage or destroy PMN leukocytes, also able to destroy adjacent cells and tissues and thus contribute to the spread of organism from local sites.

Antistreptolysin O (ASO)

Is specific neutralizing antibody produced after infection with these organisms & it appears in serum from 1 week-1month after the onset of a streptococcal infection. It combines and neutralizes the heamolytic activity of streptolysin O.

Streptolysin S

Oxygen stable non-antigenic toxic enzyme with molecular weight of 20,000 Dalton. It hemolyze red cells and phagocytic cells by direct cell to cell contact also it is responsible for the surface heterolysis observed around colonies of group A streptococci grown on blood agar plate.

Serological test

Antistreptolysin O test is used to diagnose conditions post streptococcal resulting from a streptococcal infection especially in diagnosis of rheumatic fever and glomerulonephritis when it's not possible to isolate Group A streptococci in culture (most complication develop at a stage when it is not possible to isolate groupA streptococcus in culture). The principle of the test depend on the following factors.

Antistretolysin O can react specifically with SLO and inhibits the heamolytic activity. The amount of ASO can be estimated by dilution of patient's serum in the presence of constant

amount of SLO to the point where there is still complete prevention of haemolysis. The occurrence of ASO depends on the production of SLO by streptococci in the infected host.

Commercially available test are:

- Antistreptolysin O latex slide test- used for screening a significant raise in ASO titer
- Antistreptolysin O titration test –used to determine the titer of ASO antibody.

Rapid Antistreptolysin O latex agglutination test

Principle: In the presence of ASO antibody a visible agglutination reaction will be exhibited when a serum specimen combine with latex particle coated with streptolysin O antigen.

Specimen

Clear, haemolysis free serum

Reagent & equipment required

- ✓ Latex particle coated with streptolycin O
- ✓ 0.9% NaCl solution
- ✓ Glass slide with six cells
- ✓ Applicator sticks (stirrer)
- ✓ Control reagent

  Other material required

- Timer
- Test tubes
- Pasture pipettes and rubber bulb
- Serologic pipette and safety bulb

Quality control

Positive control-a prediluted serum containing at least 200 lu/ml of ASO. This control should exhibit visible agglutination at the end of the 3-minute test period. Negative control serum a prediluted serum containing less than 100 Iu/ml of ASO. This control should exhibit a smooth or slightly granular appearance at the end of the 3-minute test period.

A positive and negative control should be tested and read concurrently with each group of patient sera.

Procedure

1. Dilute the serum by saline in 1:2
2. Label the slide for positive control dilution negative control and patient sera
3. Pipette 50μl of the controls and patient sera onto the appropriately labeled cell (well)
4. Add one drop of latex reagent to each well
5. Mix the specimen with separate applicator stick spread the mixture evenly over the well.

6. Rotate the slide for exactly 3 minutes. Examine with a bright light

Reporting

Positive – agglutination

Negative- No agglutination

Agglutination demonstrates 200 Iu/ml or more ASO. Positive results should be retested quantitatively.

A titer of 200Iu/ml or greater may be associated with rheumatic fever or glomerulonephritis.

Antistreptolysin O titration kit

In the titration test, a constant amount of streptolysin O antigen reagent is added to a series of dilutions of the patient's serum. Following a period of incubation, Group O washed human or rabbit red cells are added. The tubes are then examined for lysis of the red cells. Haemolysis occurs in those tuber in which there is insufficient antibody to neutralize the antigen.

The highest dilution of serum showing no haemolysis is the ASO titer, the titer of ASO antibody in the serum is directly proportional to the reciprocal of the serum dilution. The antistreptolysin O titer is expressed in Todd units.

Review Questions

1. Explain the property of streptolysin O and streptolysin S.

CHAPTER SIXTEEN

RHEUMATOID FACTOR

Learning Objective

At the end of this chapter, students are expected to:

- Explain the characterstics of rheumatoid factor
- Practice the serologic test for rheumatoid factor

16.0. Rheumatoid Factor

Rheumatoid factors are antibodies with specificity for antigen determinants on the F_c fragment of human or certain animal IgG. They're associated with IgG, IgM & IgA. IgG & IgM rheumatoid factors are the most common. During some disease of a joint, rheumatoid arthritis, an IgG antibody is produced following to its production the antibody become altered for unknown reason, against this altered antibody a second antibody (anti antibody) is produced which is known as antigammaglobulin antibody.

Test for Rheumatoid arthritis

Rapid latex agglutination

Principle: The test is based on the reaction between patient antibodies in the serum, known as the rheumatoid factor; and an antigen derived from gamma globulin. If rheumatoid factors are present in the serum, macroscopic agglutination will be visible when the latex reagent is mixed with the serum.

Specimen: Serum

Reagents & materials required

- Latex reagent
- Glycine saline buffer
- Capillary pipette
- Applicator stick
- Glass slide

Additional required equipment

- Timer
- Test tubes
- Serologic pipette
- Positive control
- Negative control

Procedure

1. Prepare 1:20 dilution of patient serum in glycine saline buffer (i.e. 0.1me of serum & 1.9 me of diluent) and mix the tube contents

2. Deliver one free falling drop of the diluted serum by using capillary pipette to one of the division of the slide.
3. Add one drop of positive control & one drop of negative control to the appropriately labeled division of the slide
4. Mix the latex reagent & add one drop to the patient specimen and to each of the controls.
5. Mix each specimen with a separate applicator stick over the entire area of there respective divisions on the slide
6. Tilt the slide back and forty, slowly and evenly, for 2 minutes
7. Observe for macroscopic agglutination using a direct light source.

Reporting results

Positive reaction: agglutination of the latex suspension indicates the presence of rheumatoid factor in the specimen.

Negative reaction: absence of visible agglutination.

Review Questions

1. Discuss how the rheumatoid factor is produced in the body.

Glossary

Active immunity*:* Protection acquired by deliberate introduction of Performed antibody or immune cells in to a non-immune host.

Antibody*:* Secreted immunoglobulin protein, particularly those that can bind specifically to a given antigen.

Antigen*:* A substance that reacts with antibodies or T-cell receptors.

Antiserum*:* Serum containing antibodies raised against a specific antigen

Antitoxin*:* Protective antibodies that inactivate soluble toxic protein products.

Autoimmunity*:* Immunity to self-antigens.

Bursa of Fabricius*:* The hindgut organ located in the cloaca of birds that controls the ontogeny of B-lymphocytes

Bursa equivalent*:* The hypothetical organ or organs analogous to the bursa of Fabricus in non-avian species

Cardiolipin*:* A substance derived from beef heart, probably a component of mitochondria membranes that serves as an antigenic substrate for reagin or antitreponemal antibody.

Cell mediated immunity*:* immunity in which the participation of lymphocytes and macrophages is predominant

Clone*:* A group of cells all of which are the progeny of single cells

Complement*:* A system of serum proteins that can be induced to carry out a cascade of enzymatic reactions which may lead to lysis of foreign cells and to formation of certain opsonins and inflammatory mediators.

C- reactive protein*:* An antibacterial serum protein that binds pneumococcal C-protein.

Cytokine*:* Any of a group of soluble polypeptide mediators that regulates cellular growth or function.

Effecter cell*:* Any cell that actively carries out an immunologic attack.

Hapten*:* A substance that is not immunogenic but can react with an antibody of appropriate specificity.

Lymphocytes: A mononuclear cells 7-12mm in diameter containing a nucleus with densely packed chromatin and small rim of cytoplasm.

Lymphokine*:* Any cytokine produced by lymphoid cell.

Thymus*:* A primary lymphoid organ situated in the anterior mediastinum which is required for and is the site of lymphopoiesis.

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