IMMUNOLOGY AND CELLURAR MICROBIOLOGY (DMB22)

(MSC MICROBIOLOGY)

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IMMUNOLOGY AND CELLULAR MICROBIOLOGY

M.Sc. Microbiology (Final) Paper VI.

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LESSON NO. 1.

IMMUNE RESPONSES

1.0 **Objective :** In this lesson different types of immune responses including innate immunity, acquired immunity, humoral immunity, cell mediated immunity and other types of immunity were described.

1.1. Introduction

Development of immunity : In the beginning of eleventh century Chinese determined that those who survived an attack of small Pox disease would not get the disease second time. Then after, they practiced infecting young children with small pox by either inhalation or scratching of the dried pocks on the skin of the infected persons. This method of protecting children was spread to Westward and central Asia. In 1718 Lady Mary Montagu used the same technique, to save her children from smallpox. This technique was called as 'variolation' (variola' is the latin word for small pox). In 1774 Benjamin Jesty used dried material from cowpox to variolate his children and found successful. Later 1778 Edward Jenner, a physician, confirmed the same by variolating his parents and published the results. This Jenner's technique of variolation (vacca is the latin word for cow) replaced the variolation and popularized. By 1980 smallpox had

become the first infectious disease to be eradiated from the earth by mass vaccination. In 1879 Louis Pasteur during his studies on chicken fowl cholera disease, found that aged cultures of *Pasterella multicida* , the causal agent of cholera, can even protect the chicken from disease. These aged cultures act as 'vaccine' against cholera. Pasteur, later studied the anthrax disease and found that heated cultures of *Bacillus anthrasis* can act as vaccines against the disease to protect the sheep, cattle and goat. In 1980, Emil von Behring and Shibasaburo Kitasato demonstrated that protection by vaccination is due to induced production of protective factors which they called as antibodies. Later Paul Ehrlich proved that antibodies could protect animals against toxins like recin. Similarly Pfeiffer showed that antibodies could clump and destroy *Vibrio cholerae*. Based on this, Isidone Widal demonstrated that serum from typhoid patients would make the bacteria clump whereas serum from healthy individuals can not clump- WIDAL TEST to *Salmonella typhi*. In 1894 Emile Roux demonstrated that by repeated injections of tetanus toxin to horse, antibodies against the toxin were developed in horse serum, this horse serum could be used to protect a human against tetanus for a few weeks after they have received a deep wound. This technique was known as passive immunization. In 1882 Eli Metchnikoff demonstrated that certain cells could eat foreign material, which he called phagocytes, that enter the body of invertebrates and mammals. In 1901 Emil von Behring was awarded nobel prize in medicine for his work on the production of antibodies against toxins. In 1905 Robert koch was awarded the nobel prize for his studies on tuberculosis, by the discovery of the tuberculin reaction. The nobel prize of 1908 was shared by Ehrlich and Metchnikoff for their contribution towards the development of concept that the immune system would not respond against normal body components. Karl Landsteiner was awarded a nobel prize in 1930 for his demonstration of blood groups, the complex carbohydrates found on the surface of red cells. In 1972 Rodney Porter and Gerald Edelman received the prize for demonstrating the chemical structure of antibody molecules and showed that they bound to foreign material. The 1984 nobel prize in medicine was awarded to Niels Jerne, Georges Kohler and Cesar Milstein for discovering the principles of production of monoclonal antibodies. In 1980 Donnall Thomas and Joseph Murray were awarded the prize for their pioneering work on organ transformation.

1.2. Types of immune reactions

The latin term immunis meaning 'exempt , gave rise to the english word immunity, which refers to all the mechanisms used in the body as protection against environmental agents that are foreign to the body. Immunity may be innate or acquired.

1.2.1 Innate Immunity

Innate immunity refers to the immunity response of the body due to physical and chemical barriers of the body, tissues, cells and all other mechanisms used as protection against environmental agents that are foreign to the body.

1.2.1.1. Mechanism of immunity :

a) Physical and Chemical barriers

In order to produce disease, microorganisms must enter into the body. The simplest way to avoid infection is to prevent the entry of microorganisms. It is the first line of defence against infection (the external defence system). The entry of microbes can be prevented by surface defence.

The intact skin and mucous membrane confer protection against invasion by microbes.

i) Role of skin: The skin surface consists mainly of keratin, which is indigestible by most of the microbes. Thus, as long as it is intact, is impermeable to most of the infectious agents. Most bacteria fail to survive on skin for a long time because of the inhibitory effects of saturated and unsaturated fatty acids in sweat and sebum (oily secretion of skin). Sweat also contains high concentration of salts, which are inhibitory to bacteria and fungi. Acidic pH (5.2-5.9) of the skin due to lactic acid and other acids prevents the growth of bacteria.

ii) Role of mucous membrane: Mucous membrane secretes mucus, which is a protective barrier. Microbes trapped within the adhesive mucus are removed by ciliary movements, coughing and sneezing. Mucous secretion of the respiratory, alimentary and

genitourinary tracts contains a bactericidal substance known as lysozyme, which acts on bacteria by hydrolyzing glycosidic linkages in the cell wall mucopeptides.

iii) Role of body secretion: Body secretes various fluids, which play an important role in defense mechanism. The skin secretions--sweat and sebum contain bactericidal substances. Tears contain lysozyme, which is bactericidal in nature; also the flushing action of tears makes conjuctiva free from microbes and dust particles. Saliva contains mucopolysaccharides, which inactivate bacteria and viruses. Gastric juice contains hydrochloric acid (HCl), which destroys most of the ingested bacteria and keeps stomach free from microbes. Gastric juice also contains an enzyme pepsin. Urine--the flushing action of urine eliminates bacteria from urethra. Semen is believed to contain antibacterial substances, for example, spermine.

iv) Role of commensal microflora: The microorganisms normally present in and on our body without causing any ill effects are known as commensals. Commensal microflora plays an indirect role in the defense mechanism. Some streptococci normally present in mouth produce hydrogen peroxide (H_2O_2), which is inhibitory to many other bacteria. Some of the coliform bacteria normally present in intestine produce colicins, which are inhibitory to other coliforms and shigellae. Lactobacilli normally present in vagina produce lactic acid. The resultant acidity gives protection against pyogenic infections. The propionibacteria normally present on skin produce propionic acid, which plays role in maintaining low pH. The anaerobic colon bacteria produce fatty acids with antibacterial activity. The intestinal anaerobes also prevent superinfection by coliforms during antibiotic therapy. It is also possible that the organisms of normal flora may tend to exclude pathogens by competing with them for nutrient material.

b)Tissue factors

When the infective agent penetrates the body by passing the barriers of surface defense, the tissue factors come into operation. It is the second line of defense against infection (the internal defense systems).

c) Humoral factors: Apart from specific antibodies, a variety of substances possessing antimicrobial activity are present in blood and tissue fluids. These include Lysozyme: It is a thermolabile, low molecular weight, basic protein found in high concentration in polymorphonuclear leucocytes and in most of the tissue fluids except sweat, urine, and cerebrospinal fluid (CSF). It is a mucolytic enzyme that acts on mucopeptide of the bacterial cell wall. Complement: It is a non-specific heat labile protein present in serum that has bactericidal activity. It plays an important role against pathogens invading blood and tissues. Properdin: It is a complement-like substance normally present in serum. It requires participation of components of complement and Mg++ ions for its bactericidal and antiviral activity. It is active against gram negative bacteria and shigellae in particular. Interferon: It is a non-specific antiviral agent that interferes with intracellular viral replication. It is synthesized in response to viral infections and is non-specifically active against other viruses. It increases the activity of non-specific killer cells. Phagocytin: It is a thermostable protein derived from polymorphs. It is bactericidal for many gram positive and gram negative bacteria. Other antimicrobial substances: Betalysin in serum, plakins derived from platelets, leukin from leucocytes, etc., are also active against infectious agents.

d) Cellular factors: Natural defense against microbes invading blood and tissues is medicated by phagocytic cells that engulf and digest them. Phagocytosis is the most important means of defense against microbes. There are two types of phagocytic cells - Microphages and - Macrophages. Their function is to remove the foreign particles. These cells, attracted by chemotactic mechanism, reach the sites of inflammation in large numbers and ingest the particulate material that is finally subjected to the action of the lytic enzymes present in lysosome and is digested. Microbes, for example, *Brucella* spp., lepra bacilli and tubercle bacilli which resist killing and digestion, may actively multiply inside the phagocytic cell. Phagocytic cells in such cases may actually help to disseminate infection to different parts of the body. In addition to phagocytic cells, natural killer cells and large granular lymphocytes secrete several cytotoxic proteins that can destroy viral or tumour antigens.

e) Inflammation: Tissue injury or irritation due to the entry of pathogen results in a spectrum of cellular and systemic events that leads to inflammation which is an important non-specific defense mechanism. Inflammation occurs as a result of aggregation of macrophages and microphages by chemotactic mechanisms at the site of injury. The attracted phagocytic cells engulf and destroy the pathogens. The outpouring of plasma helps to dilute the toxic products and a fibrin barrier serves to wall off the site of infection.

f) Fever: Fever is a protective defense mechanism of the body. The thermoregulatory centre in hypothalamus is sensitive to microbes and their products and reacts by increasing the body temperature, which increases circulation of blood and flushing of tissue that help to eliminate toxin through urine and sweat. Increase in body temperature may be harmful to invading microbes and in some instances may destroy the pathogens. It stimulates the production of interferon that helps in recovery of viral infection.

1.2.2. Acquired immunity

It is defined as an immunity specific for a particular disease, which an individual acquires during the course of his life. As it is specific for a particular disease, it is also known as specific immunity. It is of two types a) Active immunity b) Passive immunity

1.2.2.1. Active immunity: It is the resistance developed by an individual in response to the microbes or their products (antigenic stimulus). The entry of antigen results in activation of immunocompetent cells producing antibodies (humoral/antibody mediated immunity) or activated T cells (cell mediated immunity). Active immunity requires a considerable time (latent period) for its development (weeks or months) but once developed, persists for long duration and may last for years. Active immunity is associated with immunological memory. The memory cells produced after the first entry of antigen retain the memory for long periods and give rapid and vigorous response when the same antigen enters subsequently (secondary response). Active immunity is of two types a) Naturally acquired active immunity. b) Artificially acquired active immunity.

a) Naturally acquired active immunity: Immunity that an individual develops as a result of natural contact with a pathogenic microbe. This contact may result in a major invasion with clinical disease are a minor invasion without clinical disease (inapparent or subclinical infection). Following infection, the patient, in most cases, will be resistant to further infection by the same pathogen for a period, which is different in different diseases. In some diseases like influenza, common cold, gonorrhoea and staphylococcal infection, the immunity lasts for short duration. While in other infections such as diphtheria, smallpox, measles, yellow fever, etc., it lasts for long duration and may persist for life. In general, immunity following bacterial infection is less permanent than immunity following viral infection. In syphilis, a special type of immunity known as 'premunition' or 'infection immunity' is seen, i.e., immunity to reinfection persists as long as the original infection remains active. Once the disease is cured and the organisms are eliminated from the body, the patient again becomes susceptible to the reinfection by *Treponema pallidum.* This accounts for the pingpong syphilis in sailors who acquire infection at one port, take treatment at next port, to get infection again at the next port.

b) Artificially acquired active immunity: Immunity, which an individual acquires as a result of artificial inoculation of microbes or their products (immunization with microbes or their products). The degree of immunity produced is same as that of natural infection. For immunization, vaccines or their products are used. Vaccines are preparations containing live or killed microbes. Examples are -

1. Live attenuated vaccines.
   a) BCG for tuberculosis. } Bacterial
   b) Typhoral for typhoid.
   c) Sabin oral vaccine for polio. } Viral
   d) MMR for mumps, measles and rubella.

2. Killed vaccines
   a) TAB for enteric fever. } Bacterial
   b) Cholera vaccine.

c) Salk vaccine for polio. } Viral
d) Rabies vaccine.

3. Microbial products.
   a) Diphtheria toxoid.
   b) Tetanus toxoid.

1.2.2.2. Passive immunity: The resistance, which is induced by transfer of preformed antibodies against microbes or their products in another host, is known as passive immunity. Here, the immune system does not take any active part in the development of immunity. The passive immunity is rapidly established and the protective mechanisms come into force immediately. It is useful in certain situations like gas gangrene, tetanus, diphtheria and snake bite where the immediate protection is required. Passive immunity is of two types: a) Naturally acquired passive immunity and b) Artificially acquired passive immunity.

a) Naturally acquired passive immunity: The newborn babies are normally devoid of acquired active immunity but are resistant to number of infections such as measles, chickenpox, diphtheria and scarlet fever. This resistance is due to passive transfer of antibody from mother to foetus and lasts for three to four months.

b) Artificially acquired passive immunity: Passive immunity can be acquired artificially by injection of antibodies. The agents used for this purpose are hyperimmune sera of animal or human origin, convalescent sera and pooled human gammaglobulin. This immunity lasts for short duration, for example, the diphtheria antitoxin has a half life of seven days. This procedure is used for: Treatment and also for prophylaxis and particularly indicated in clinical emergency for providing immediate and temporary protection in a non-immune host. The suppression of active immunity when it is injurious as in Rh negative women with Rh positive babies. For passive immunization, the hyperimmune sera of horse, sheep, goat, rabbit, guinea pigs or human beings are used. The serum may be antitoxic, antibacterial or antiviral.

1.2.3. Humoral Immunity

Humoral immunity is mediated by serum antibodies, which are the proteins secreted by the B cells. B cells are initially activated to secrete antibodies after the binding of antigens to specific membrane immunoglobulin (Ig) molecules (B cell receptors), which are expressed by these cells. Antibodies are a heterogenous mixture of serum globulins, all of which share the ability to bind individually to specific antigens.

All immunoglobulin molecules have common structural features, which enable them to do two things (1) recognize and bind specifically to a unique structural entity on an antigen (namely, the epitope) and (2) perform a common biologic function after combining with the antigen. Basically, each immunoglobulin molecule consists of two identical light (L) chains and two identical heavy chains (H), linked by disulfide bridges. On the basis of differences in their H chains, these molecules are divided into five major classes IgG, IgM, IgA, IgE and IgD each of which has several unique biological properties. It is important to note that antibodies in all five classes may possess precisely the same specificity against an antigen (antigen-binding sites), while at the same time having different functional (biological effector) properties.

Another important element involved in humoral immunity is the complement system. The reaction between antigen and antibody serves to activate this system, which consists of a series of serum enzymes, the end result of which is lysis of the target or enhanced phagocytosis (Ingestion of the antigen) by phagocytic cells. The activation of complement also results in the recruitment of highly phagotrophic polymorphonuclear (PMN) cells, which constitute part of the innate immune system. These activities maximize the effective response made by the humoral of immunity against invading antigens.

1.2.4. Cell Mediated Immunity

CMI is the specific immune response mediated by sensitised T-cells independent of Ab. Certain microorganisms such as Bacteria---tubercle and leprosy bacilli. Viruses---small pox and measles. Parasites---Toxoplasma and Leishmania. Fungi---Histoplasma and Blastomyces. These organisms are intracellular pathogens, which have the ability to multiply within the host cells. Hence, Abs are not effective against such pathogens. Immunity against these pathogens is masterminded by the T-lymphocytes. Such an immune response, which involves the interaction of cells of the immune system with the Ag, is known as CMI.

Induction of CMI: The nature of antigenic stimulus is important in the induction of CMI. The intracellular parasites are the best stimulators. The T-cells possess specific receptors (TCR) on their surface for Ag. The CMI is initiated by the binding of Ag with an Ag receptor on T-lymphocyte. The Ag may react directly or it may be presented by macrophage. When specific Ag reacts with specific receptor on T-cells, they undergo proliferation and a sequence of morphological and biochemical events occurs. The cell membrane of T-cell becomes activated and the signal is transmitted to interior of the cell where the nucleus becomes depressed and the cell transforms into a large blast cell--blast transformation. DNA, RNA and protein synthesis is increased. This interaction finally results in blast transformation, clonal proliferation and differentiation. The result of these events are - The generation of Th and Ts cells for T-T and T-B interactions ; generation of cytotoxic T-cells ; generation of lymphokine producing T-cells ; generation of memory cells.

Proliferated T-lymphocytes provide two major effector mechanisms. A. The release of biologically active soluble factors called lymphokines (cytokines).B. The generation of cytotoxic T-cells of artificial active immunization, it may be possible to eradicate communicable diseases like diphtheria, polio, etc.

Detection of CMI: Several tests are now available for detection of CMI. Skin test was the only method available till recently. A number of in vitro methods are now available.

These include Lymphocyte transformation test- in which there is a transformation of cultured sensitised T-lymphocytes on contact with specific Ag to blast cells---evidenced by enhanced DNA synthesis. Target cell destruction---in which there is a killing of cultured cells by lymphocytes sensitised against them. Migration inhibiting factor test---it is the most commonly used test for detection of CMI. In this test, macrophages packed in a capillary tube when placed in a tissue culture medium in a chamber, macrophages migrate out and spread over the galls walls of the chamber and form a lacy fan like appearance. If macrophages are from sensitised guinea pig, the addition of Ag to the culture chamber will inhibit the migration. The test has been adopted for clinical use by incubating human peripheral leucocytes in capillary tubes to culture chambers. When specific antigen is added, the migration of leucocytes is prevented. By comparing with the control test, a semiquantitative assessment of the migration inhibition is possible.

Scope of CMI: CMI plays an important role in Immunity against infectious diseases caused by obligate and facultative intracellular pathogens. Delayed hypersensitivity. Transplantation immunity and graft vs host reaction. Immunological surveillance and immunity against cancer (tumour rejection). Pathogenesis of certain autoimmune diseases such as thyroiditis, encephalomyelitis, etc.

1.2.5. Other types of immunity

1.2.5.1. Local immunity: Certain microbes infect only certain groups of cells and selective tissues. Resistance against such pathogens depends on the immunity of the corresponding cells and tissues. This is known as local immunity. For example, in poliomyelitis and influenza, systemic immunity developed as a result of active immunization with killed vaccines neutralizes the viruses when they enter the blood stream but the multiplication of viruses at the site of entry (gut mucosa in polio and respiratory tract in influenza) cannot be prevented. The local multiplication of the virus can be prevented by the local immunity acquired as a result of infection or immunization with the live oral polio vaccine and intranasal influenza vaccine. A special type of antibody, IgA, plays an important role in local immunity. This IgA antibody, known as secretory IgA, is produced locally by plasma cells present on mucosal surfaces or in

secretory glands. The IgA is the principal component in various body secretions such as mucus of respiratory, intestinal, urinary, and genital tracts, tears saliva, and milk.

1.2.5.2. Herd immunity: It is the overall level of immunity in a community and is important in the control of outbreaks. When herd immunity is low, an infectious disease may spread rapidly and may be severe in nature. When herd immunity is high, the infectious disease spreads less rapidly and is of mild form. By developing high level of herd immunity by means

1.2.6. Primary immune response

When Ag enters for the first time (priming dose), the body gives primary response (Fig. 1.1). Immediately after the priming dose of an Ag, no Ab is detected in the serum. This period is known as latent period. After this, an active biosynthesis of Ab occurs during a log phase. The Ab concentration in serum remains constant during a plateau or steady state. Finally, a declined phase is observed in which catabolism is greater than synthesis.

Thus, the primary response is S-Slow, S-sluggish, S-short lived ; with long lag phase of 5-7 days; low titres of Abs that persist for short duration and type of Ab - IgM of low affinity, low avidity and differ in specificity (Table 1.1).

Table 1.1 Difference between Primary and Secondary response

Primary Response	Secondary Response
1. Slow, sluggish, short lived	Prompt, powerful, prolonged
2. Long latent phase	Short or negligible latent phase
3. Rate of Ab synthesis – low	Rate of Ab synthesis – high
4. Type of Ab – IgM	Type of Ab – IgG
5. Peak Ab titre – low	Peak Ab titre – high
6. Persistence of Ab titre – short period	Persistence of Ab titre – long period

7. Affinity of Ab – low	Affinity of Ab – high
8. Memory cells – few	Memory cells – many
9. Dose of Ag – high	Dose of Ag – low

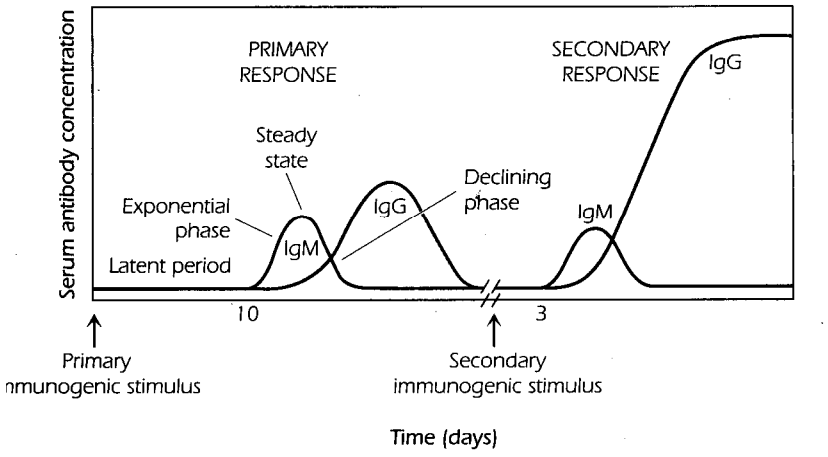

Fig. 1.1. The kinetics of an antibody response

1.2.7. Secondary immune response:

When the same Ag enters for the second time (booster dose), after weeks, months or even after years, the body gives secondary response. Following the booster dose, there is a markedly enhanced response that is characterized by the accelerated appearance of immunocompetent cells and Ab.

Thus, the secondary response (anamnestic or recall) is P-prompt, P-powerful, P-prolonged ; with short or negligible latent phase of (2-3 days) ; high levels of Abs, lasting for long period and type of Ab involved is IgG of increased affinity, avidity and specificity.

If the specific Ab is present in the serum at the time of booster dose, the Ab disappears more rapidly than in the decline phase of the primary response. This is negative phase which is due to the combination of the Ab with newly injected Ag. If the dose of Ag is too small, no secondary response because of Ag-Ab complex formation.

These differences in primary and secondary response are due to the number of responding B-cells. In the primary response, only few of the B-cells are converted into Ab producing B-cells and large number of B cells are converted into memory cells. In the secondary response, all B-cells are converted by Ag into Ab producing B-cells.

Memory cells are longer lasting cells, which are able to respond same Ag when it enters for the second time and help to B-cells to produce Abs in high titre. The life of memory cells is three years or more, e.g., tetanus toxoid, can evoke a powerful secondary response after 20 years also.

IgM to IgG switch: Immune response is genetically controlled. An individual cell first produces IgM and then IgG, IgA, IgD and IgE. This is because of genes and genetic control.

At first, there is switching of μ gene so that IgM is produced, then there is looping out of μ gene, so that information is transmitted to γ gene to produce IgG. In this way, IgM antibodies are synthesized first and then IgG, IgA, IgD and IgE.

The capacity to make a secondary response may persist for a long time (years in humans), and it provides an obvious selective advantage for individuals who survive the first contact with an invading pathogen. Establishment of this memory for generating a specific response is, of course, the purpose of public health immunization programs.

1.3. Summary

Immunity, refers to all the mechanisms used in the body as protection against environmental agents that are foreign to the body. Immunity may be innate or acquired. Innate immunity refers to the immunity response of the body due to physical and chemical barriers of the body, tissues, cells and all other mechanisms used as protection against environmental agents that are foreign to the body. Acquired immunity is the immunity that an individual acquires during the course of his life for a particular disease. As it is specific for a particular disease, it is also known as specific immunity. It is of two types a) Active immunity b) Passive immunity. Active immunity is the resistance developed by an individual in response to the microbes or their products (antigenic

stimulus). Passive immunity is a type of immunity that is induced by transfer of preformed antibodies against microbes or their products in another host. Humoral immunity is mediated by serum antibodies, which are the proteins secreted by the B cells after the binding of antigens to specific membrane immunoglobulin. Immune response involving the interaction of cells of the immune system with the Ag, is known as Cell mediated immunity. The response of the body for the first time entry of Ag is called primary immune response. When the same Ag enters for the second time (booster dose), after weeks, months or even after years, the body gives secondary response. In the primary response, only few of the B-cells are converted into Ab producing B-cells and large number of B cells are converted into memory cells. In the secondary response, all B-cells are converted by Ag into Ab producing B-cells.

1.4. Model Questions

Write an essay on innate and acquired immunity reactions.

Give an account on humoral and cell mediated immunity reactions

Write short notes on

a) development of immunology

b) cell involved in innate immunity

c) different types of immune reactions

d) different types of acquired immune reactions

e) primary and secondary immune response

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LESSON NO. 2.

COMPONENTS OF IMMUNE SYSTEM–I

CELLS

2.0. Objective : In this lesson, structure and functions of cellular components of immune system namely B cells, T Cells , mononuclear phagocytic cells, granulocytes and other cells were described.

2.1. Introduction

Lymphocytes are the central cells of the immune systems, responsible for acquired immunity and the immunologic attributes of diversity, specificity, memory, and self/non self recognition. The other types of white blood cells play important but often ancillary roles, engulfing and destroying microorganisms, presenting antigens, and secreting cytokines.

Lymphocytes constitute 20%-40% of the body's white blood cells and 99% of the cells in the lymph. There are approximately 10^{11} (range depending on body on body size and age: $10^{10} \sim 10^{12}$) Lymphocytes in the human body. These Lymphocytes continually circulate

in the blood and lymph and are capable of migrating into the tissue spaces and lymphoid organs, thereby integrating the immune system to a high degree.

The Lymphocytes can be broadly subdivided into three population-B cells, T cells, and null cells-on the basis of function and cell-membrane components. The null population of lymphocytes does not express surface markers typical of B or T cells.

It consists mostly of large, granular lymphocytes which are the natural killer cells (NK cells). Resting B and T lymphocytes are small, motile, non phagocytic cells, which cannot be distinguished morphologically. B and T lymphocytes that have not interacted with antigen-referred to as naïve, or unprimed – are resting cells in the G_0 phase of the cell cycle (known as small lymphocytes). These cells are only about 6μm in diameter; their cytoplasm forms a barely discernible rim around the nucleus. Small lymphocytes have densely packed chromatin, few mitochondria, and a poorly developed endoplasmic reticulum and Golgi appar
atus. The naïve lymphocyte is generally thought to have a short life span. Interaction of small lymphocytes with antigen, in the presence of certain cytokines, induces these cells to enter the cell cycle by progressing from G_0 onto G_1 and subsequently into S,G_2, and M. As they progress through the cell cycle, lymphocytes enlarge into 15μm-diameter blast cells, called lymphoblasts; these cells have a higher cytoplasm: nucleus ratio and more organellar complexity than small lymphocytes .

Lymphoblasts proliferate and eventually differentiate into effector cells or into memory cells. Effector cells function in various ways to eliminate the antigen. These cells have short life spans, generally ranging from a few days to a few weeks. Plasma cells-the antibody-secreting effector cells of the B-cell lineage-have a characteristic cytoplasm that contains abundant endoplasmic reticulum (to support their high rate of protein synthesis) arranged in concentric layers and also many Golgi vesicles. The effector cells of the T-cell lineage include the cytokine-secreting T helper cell TH cell) and the cytotoxic T lymphocyte Tc or, sometimes, CTL). Some of the progeny of B and T lymphoblasts differentiate into memory cells. The persistence of this population of cells is responsible

for life-long immunity to many pathogens. Memory cells look like small lymphocytes but can be distinguished from naïve cells by the presence or absence of certain cell-membrane molecules.

Different lineages or maturational stages of lymphocytes can be distinguished by their expression of membrane molecules recognized by particular monoclonal antibodies. All of the monoclonal antibodies that react with a particular membrane molecule are grouped together as a cluster of differentiation (CD). Each new monoclonal antibody that recognizes a leukocyte membrane molecule is analyzed for whether it falls within a recognized CD designation; if it does not, it is given a new CD designation reflecting a new membrane molecule. Although the CD nomenclature was originally developed for the membrane molecules of human leukocytes, the homologous membrane molecules of other species, such as mice, are also commonly referred to by the same CD designations.

2.2. B Lymphocytes

The B lymphocyte derived its letter designation from its site of maturation, in the bursa of Fabricius in birds; the name turned out to be apt, for bone marrow is its major site of maturation in a number of mammalian species, including humans and mice. Mature B cells are definitively distinguished from other lymphocytes by their synthesis and display of membrane-bound immunoglobulin (antibody) molecules, which serve as receptors for antigen. Each of the approximately 1.5×10^5 molecules of antibody on the membrane of a single B cell has an identical binding site for antigen. Among the other molecules expressed on the membrane of mature B cells are -

- B220 (a from of CD 45, CD45R) is frequently used as a marker for B cells and their precursors however, unlike antibody, it is not found uniquely on B-lineage cells.
- Class II MHC molecules permit the B cell to function as an antigen-presenting cell (APC).
- CRI (CD35) and CR2 (CD21) are receptors for certain complement products.
- Fcγ RII (CD32) is a receptor for IgG, a type of antibody.

- B7-1 (CD80) and B7-2 (CD86) are molecules that interact with CD28 and CTLA-4, important regulatory molecules on the surface of different types of T cells, including TH cells.
- CD40 is a molecule that interacts with CD40 ligand on the surface of helper T cells. In most cases this interaction is critical for the survival of antigen-stimulated B cells and for their development into antibody-secreting plasma cells or memory B cells.

Interaction between antigen and the membrane-bound antibody on a mature naïve B cell, as well as interactions with T-cells and macrophages, selectively induces the activation and differentiation of B-cells clones of corresponding specificity. In this process, the B cell divides repeatedly and differentiates over a 4 to 5 day period, generating a population of plasma cells and memory cells. Plasma cells, which lack membrane-bound antibody, synthesize and secrete one of the five classes of antibody. All clonal progeny from a given B cell secrete antibody molecules with the same antigen-binding specificity. Plasma cells are terminally differentiated cells, and many die in 1 or 2 weeks.

Maturation of B cells: They originate from precursor cells from the yolk sac, foetal liver and bone marrow. During the maturation process, the pre B-cell is programmed to produce only one class or subclass of Ig after a switch from initial IgM production. On the basis of immunoglobulin, which is programmed to synthesize, B-lymphocytes can be subdivided into nine different subsets---IgM, IgD, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 and IgE.

The immature B-cells with IgM on the cell surface are known as virgin B-cells, which are immunologically competent but have not had contact with Ag. The virgin B-cells migrate to the periphery (spleen and lymphoid tissues) where they express IgD on their surface and one of the other Ig classes like, IgM, IgG, IgA or IgE. By reassortment of Ig genes, B-cells develop the capacity to produce Ig molecules.

On contact with specific Ag, the B-cell undergoes clonal proliferation. Some of the activated B-cells are converted into memory cells which are responsible for the recall

phenomenon on subsequent contact with the same Ag and majority of the activated B-cells, are converted into Ab producing plasma cells.

Distribution of B-Cells: Approximately 30% of the lymphocytes circulating in the blood are B-cells. Their distribution in various organ and tissues is: Peripheral blood---15-30%, Lymph nodes---30-35%, Bone marrow---75%, Spleen---55-60%, Tonsillar lymphocytes---50%.

Functions of B-cells: B-lymphocytes are the mediators of humoral immune response. which produce Abs.

2.2.1. Subsets of B-lymphocytes: B-cells also exist in different subpopulations (subsets) based on the type of immunoglobulin synthesized by them. Thus, the different subsets are those that selectively form the immunoglobulin classes, e.g., IgM producing B-cells, IgG producing B-cells and so on.

2.2.2. Activation of B-cells: Ig present on the surface of B-cell behaves as the specific receptor for Ag. When Ag enters into the body, it reacts with B-cell with appropriate specificity. This interaction stimulates B-cell to undergo blastoid transformation and to get converted into plasma plasts (clone formation) and finally into plasma cells.

Each B-cell possesses the genetic instrument to produce Ab of unique Ag specificity as a membrane receptor. Once the signal is received, B-cells are differentiated into plasma cells, which produce and secrete Abs.

Plasma cells: Antigenically stimulated B-cells undergo blast transformation to form plasma cells. These are twice the size of small lymphocyte The properties of mature plasma cells are: oval in shape ;.with small, eccentric, oval nucleus having radially arranged chromatin around the periphery that gives the appearance of a clock face or cart wheel. Cytoplasm contains a well developed Golgi apparatus and abundant endoplasmic reticulum. The life-span of these cells is two or three days. The aggregates of Ig, called

Russell bodies are seen sometimes in the endoplasmic reticulum. These cells are distributed in germinal centers of lymph nodes, spleen and diffused lymphoid tissue of alimentary and respiratory tracts. Plasma cells are the major Ab producing cells but lymphocytes and lymphoblasts may also produce Abs to a certain extent.

Function: Plasma cell is an antibody producing machinery. It can produce an Ab of a single specificity either IgM or IgG or IgA.

2.3. T Lymphocytes

T lymphocytes derive their name from their site of maturation in the thymus. Like B lymphocytes, these cells have membrane receptors for antigen. Although the antigen-binding T-cell receptor is structurally distinct from immunoglobulin, it does share some common structural features with the immunoglobulin molecule, most notably in the structure of its antigen-binding site. Unlike the membrane-bound antibody; on B cells, the T-cell receptor (TCR) does not recognize free antigen. Instead the TCR recognizes only antigen that is bound to particular classes of self-molecules. Most T cells recognize antigen only when it is bound to a self-molecule encoded by genes within the major histocompatibility complex (MHC). Thus, a fundamental difference between the humoral and cell-mediated branches of the immune system is that the B cell is capable of binding soluble antigen, whereas the T cell is restricted to binding antigen displayed on self-cells. To be recognized by most T cells, this antigen must be displayed together with MHC molecules on the surface of antigen-presenting cells or on virus-infected cells, cancer cells, and grafts. The T-cell system has developed to eliminate these altered self-cells, which pose a threat to the normal functioning of the body.

2.3.1. Maturation of T-lymphocytes: T-cells originate from precursor cells from the yolk sac, foetal liver and bone marrow and migrate to the thymus and mature there. Several subsets of the T-cells arise during maturation process, each one is responsible for specific function. The earliest identifiable T-cells are the $CD7^{+}$ pro T cells, which acquire CD2 on entering the thymus. They synthesize CD3 in the cytoplasm and become pre T-cells. They also synthesize T-cell receptor (TCR). TCR is a heterodimer of

lipoprotein chains, which along with CD3 acts as the Ag recognition unit like immunoglobulin on the surface of B-cells. TCR occurs as two pairs of glycoprotein chains either α β or γ δ. In majority of T-cells it is α β TCR. It is closely associated with CD3 Ag as transmembrane proteins. Both the TCR and CD3 molecules are analogous to immunoglobulins and each possesses variable and constant regions. The variable regions of α and β chains constitute the Ag binding site and non-covalently linked CD3 molecule is believed to act as a signal transducer that transduces Ag recognition signal to the interior of the cell. T-cells also develop MHC restriction, so that each T-cell after antigenic stimulation carries unique Ag specificity.

2.3.2. Distribution of T-lymphocytes: T-lymphocytes are richly distributed in thymus, lymph nodes and peripheral blood. Their distribution in Peripheral blood is 55-75%, Lymph nodes - 60-75%, Spleen - 25-45%, Bone marrow - 10%, Thymus - more than 75%.

2.3.3. Functions of T-cells: a) Mediate CMI through the production of cytokines. b) Can directly act on and destroy virally infected host cells, tumour cells and foreign cells (cytotoxic effect). c)Act as regulatory cells that modulate the activity of other T-cells, B-cells or macrophages. d)Regulation can be in the form of help or suppression.

2.3.4. Types of T-cells:

Like B cells, T cell express distinctive membrane molecules. All T-cell subpopulation express the T-cell receptor, a complex of polypeptides that includes CD3; and most can be distinguished by the presence of one or the other of two membrane molecules, CD4 and CD8. In addition, most mature T cells express the following membrane molecules:

CD28, a receptor for the co-stimulatory B7 family of molecules present on B cells and other antigen-presenting cells.

CD45, a signal-transduction molecule

T cells that express the membrane glycoprotein molecule CD4 are restricted to recognizing antigen bound to class II MHC molecules, whereas T cells expressing CD8, a

dimeric membrane glycoprotein, are restricted to recognition of antigen bound to class I MHC molecules. Thus the expression of CD4 versus CD8 corresponds to the MHC restriction of the T cell. In general, expression of CD4 and of CD8 also defines two major functional subpopulations of T lymphocytes. $CD4^+$ T cells generally function as T helper (TH) cells and are class-II restricted; $CD8^+$ T cells generally function as T cytotoxic (TC) cells and are class-I restricted. Thus the ratio of TH to TC cells in a sample can be approximated by assaying the number of $CD4^+$ and $CD8^+$ T cells. This ratio is approximately 2:1 in normal human peripheral blood, but it may be significantly altered by immunodeficiency diseases, autoimmune diseases, and other disorders.

TH cells are activated by recognition of an antigen-class II MHC complex on an antigen-presenting cell. After activation, the TH cell begins to divide and gives rise to a clone of effector cells, each specific for the same antigen-class II MHC complex. These TH cells secrete various cytokines, which play a central role in the activation of B cells, T cells, and other cells that participate in the immune response Changes in the pattern of cytokines produced by TH cells can change the type of immune response that develops among other leukocytes. The TH 1 response produces a cytokine profile that supports inflammation and activates mainly certain T cells and macrophages, whereas the TH2 response activates mainly B cells and immune responses that depend upon antibodies. TC cells are activated when they interact with an antigen-class I MHC complex on the surface of an altered self-cell (e.g., a virus-infected cell or a tumor cell) in the presence of appropriate cytokines. This activation, which results in proliferation, causes the TC cell to differentiate into an effector cell called a cytotoxic T lymphocyte (CTL). In contrast to TH cells, most CTLs secrete few cytokines. They cause destruction of target cells by releasing molecules known as lymphotoxins and perforin.

Another subpopulation of T lymphocytes-called T suppressor (TS) cells-has been postulated which constitute 25-40% of circulating T-lymphocytes. It is clear that some T cells help to suppress the humoral and the cell-mediated branches of the immune system by blocking Th activity or by acting directly on B-cells, but the actual isolation and cloning of normal TS cells is a matter of controversy and dispute among immunologists.

For this reason, it is uncertain whether TS cell do indeed constitute a separate functional subpopulation of T cells. Some immunologists believe that the suppression mediated by T cells observed in some systems is simply the consequence of activities of TH or TC subpopulations whose end results are suppressive

Other types

T-regulator cells (Tr): Tr cells are the regulator cells, which regulate the activity of Th and Ts cells.

Delayed hypersensitivity T-cells (TD-cells): T-cells responsible for delayed type of hypersensitivity reactions are known as TD-cells. They are indistinguishable from Th-cells on the basis of surface markers. It is believed that TD-cells are one type of T-helper cells - Th1. They possess CD4 markers and secrete different lymphokines (e.g., γ interferon), which are responsible for inflammatory response of delayed hypersensitivity. These cells also secrete growth factors, which are believed to regulate lymphocyte activity.

2.4. Null cells:

A small group of lymphocytes, called null cells, in the peripheral blood do not express the membrane molecules and receptors that distinguish T- and B-cell lineages. These cells also fail to synthesize immunoglobulin and incorporate it into their plasma membrane. Because these cells do not produce antigen-binding receptor, they lack precise immunologic specificity and memory. Most members of the null cell population are large, granular lymphocytes called natural killer (NK) cells: these cells constitute 5%-10% of the lymphocytes in human peripheral blood.

The natural killer cell was first described in 1976, when it was shown that certain null cells display cytotoxic activity against a wide range of tumor cells in the absence of any previous immunization with the tumor. NK cells were subsequently shown to play an important role in host defense both against tumor cells and against cells infected with some, though not all, viruses. NK cells can recognize potential target cells in two

different ways. In some cases, an NK cell employs NK cell receptors to distinguish abnormalities, notably a reduction in the display of class I MHC molecules and the unusual profile of surface antigens displayed by some tumor cells and cells infected by some viruses. Another way in which NK cells recognize potential target cells depends upon the fact that some tumor cells and cells infected by certain viruses display antigens against which the immune system has made and antibody response, so that antitumor or antiviral antibodies are bound to their surfaces. Because NK cells express CD16, a membrane receptor for the carboxyl-terminal end of the IgG molecule, called the Fc region, they can attach to these antibodies and subsequently destroy the targeted cells. This is an example of a process known as antibody-dependent cell-mediated cytotoxicity (ADCC).

Several observations suggest that NK cells play an important role in host defense against tumors. For example, in humans the Chediak-Higashi syndrome- an autosomal recessive disorder-is associated with impairment in neutrophils, macrophages, and NK cells and an increased incidence of lymphomas. Likewise, mice with an autosomal mutation called beige lack NK cells; these mutants are more susceptible than normal mice to tumor growth following injection with live tumor cells.

There has been growing recognition of a special cell type, the NK1-T cell, that has some of the characteristics of both T cells and NK cells. Like T cells, have T cell receptors (TCRs). Unlike most T cells, the TCRs of NK1-T cells interact with MHC – like molecules called Cd1 rather than with class I or class II MHC molecules. Like NK cells, they have variable levels of CD 16 and other receptors typical of NK cells, and they can kill cells. A population of triggered NK1-T cells can rapidly secrete large amounts of cytokines needed to support antibody production by B cells as well as inflammation ; the development and expansion of cytotoxic T cells. Some immunologists view this cell type as a kind of rapid response system that has evolved to provide early help while conventional TH-Cell responses are still developing.

2.5. Phagocytic cells

Engulfment and digestion of foreign particle by a single cell is known as phagocytosis. The process of phagocytosis is the most important means of defense mechanism against microorganisms. It is a part of non-specific defense mechanism. The inactivation, removal, and disposal of microorganism is done by phagocytic cell , often with the help of Ab and complement. Phagocytic cells recognize foreign particles with the help of specific Abs and digest them with the help of complement factors. The phagocytic cells were first described by Metchnikoff (1883).

Role of phagocytic cells: The primary role is the phagocytosis--engulfment and digestion of foregin particles. Also participates in the development of specific immune response, e.g., trapping of Ag by macrophages and its presentation to lymphocytes in optimal concentration. Macrophages also participate in antitumour activity and graft rejection. Microphages do not play any significant role in specific immune response, however, they participate in inflammation, opsonisation, hypersensitivity reactions and immunity against parasitic infections.

2.5.1. Types of phagocytic cells

In man, phagocytosis is carried out primarily by mononuclear macrophages of blood and tissue, neutrophils and to a lesser extent eosinophils. The cells with less or no phagocyte activity but play an important role in immune reactions are basophils, mast cells and dendritic cells.

2.5.1.1. Mononuclear macrophages :

The mononuclear phagocyte system consists of monocytes circulating in the blood and macrophages in the tissues. During hematopoiesis in the bone marrow, granulocyte-monocyte progenitor cells differentiate into promonocytes, which leave the bone marrow and enter the blood, where they further differentiate into mature monocytes.

Monocytes circulate in the bloodstream for about 8 h, during which time they enlarge; then they migrate into the tissues and differentiate into specific tissue macrophages.

Differentiation of monocyte into a tissue macrophage involves a number of changes: The cell enlarges five-to ten-fold; its intracellular organelles increase in both number and complexity; and it acquires increased phagocytic ability, produces higher levels of hydrolytic enzymes, and begins to secrete a variety of soluble factors. Macrophages are dispersed throughout the body. Some take up residence in particular tissues, becoming fixed macrophages, whereas others remain motile and are called free, or wandering, macrophages. Free macrophages travel by amoeboid movement throughout the tissues. Macrophage-like cells serve different functions in different tissues and are named according to their tissue location: Alveolar macrophages in the lung ; Histocytes in connective tissues ; Kupffer cell in the liver ; Mesangial cells in the kidney ; Microglial cells in the brain ; and Osteoclasts in bone. Although normally in a resting state, macrophages are activated by a variety of stimuli in the course of an immune response. Phagocytosis of particulate antigens serves as an initial activating stimulus. However, macrophage activity TH cells, by further enhanced by cytokines secreted by activated TS cells, by mediators of the inflammatory response, and by components of bacterial cell walls. One of the most potent activators of macrophages is interferon gamma (IFNγ) secreted by activated TH cells.

Activated macrophages are more effective than resting ones in eliminating potential pathogens, because they exhibit greater phagocytic activity, an increased ability to kill ingested microbes, increased secretion of inflammatory mediators, and an increased ability to activate T cells. In addition activated macrophages, but not resting ones, secrete various cytotoxic protein that help them eliminate a broad range of pathogens, including virus-infected cells, tumor cells, and intracellular bacteria. Activated macrophages also express higher levels of class II MHC molecules, allowing them to function more effectively as antigen-presenting cells. Thus, macrophages and TH cells during the immune response facilitate each other's activation.

2.5.1.2. Granulocytic cells (Microphages): These are small non-dividing polymorphonuclear leucocytes or granulocytes present in blood. Morphologically, there

are three types of cells, which participate in immunological reactions. These include the neutrophils (45-60%), the eosinophils (1-3%) and the basophils (0.3%) of the total leucocyte count. The neutrophils and to a lesser extent, the eosinophils are phagocytic. They contain granules and a wide range of bactericidal substances. They originate in the bone marrow from stem cells, undergo maturation and finally released into circulation. They are short lived cells with half-life of two days in circulation and few hours in tissue after penetration.

The granulocytes are classified as neutrophils, eosinophils, or basophils on the basis of cellular morphology and cytoplasmic staining characteristics. The neutrophil has a multilobed nucleus and a granulated cytoplasm that stains with both acid and basic dyes; it is often called a polymorphonuclear leukocyte (PMN) for its multilobed nucleus. The eosinophil has a bilobed nucleus and a granulated cytoplasm that stains with the acid dye eosin red (hence its name). The basophil has a lobed nucleus and heavily granulated cytoplasm that stains with the basic dye methylene blue. Both neutrophils and eosinophils are phagocytic, whereas basophils are not. Neutrophils, which constitute 50%-70% of the circulating white blood cells, are much more numerous than eosinophils (1%-3%)or basophils (<1%).

2.5.1.2.1. Neutrophils

Neutrophils are produced by hematopoiesis in the none marrow. They are released into the peripheral blood and circulate for 7-10 h before migrating into the tissues, where they have a life span of only a few days. In response to many types of infections, the bone marrow releases more than the usual of infections, the neutrophils and these cells generally are the first to arrive at a site of inflammation. The resulting transient increase in the number of circulating neutrophils, called leukocytosis, is used medically as an indication of infection.

Movement of circulating neutrophils into tissues, called extravasation, takes several steps: the cell first adheres to the vascular endothelium, then penetrates the gap between adjacent endothelial cells lining the vessel wall, and finally penetrates the vascular basement membrane, moving out into the tissue spaces. A number of substances generated in an inflammatory reaction serve as chemotactic factors that promote

accumulation of neutrophils at an inflammatory site. Among these chemotactic factors are some of the complement components of the blood-clotting system, the several cytokines secreted by activated TH cells and macrophages.

Like macrophages, neutrophils are active phagocytic cells. Phagocytosis by neutrophils is similar to that described for macrophages, except that the lytic enzymes and bactericidal substances in neutrophils are contained within primary and secondary granules. The larger, denser primary granules are a type of lysosome containing peroxidase, lysozyme, and various hydrolytic enzymes. The smaller secondary granules contain collagenase, lactoferrin, and lysozyme. Both primary and secondary granules fuse with phagosomes, whose contents are then digested and eliminated much as they are in macrophages.

Neutrophils also employ both oxygen-dependent and oxygen-independent pathway to generate antimicrobial substances. Neutrophils are in fact much more likely than macrophages to kill ingested microorganisms. Neutrophils exhibit a larger respiratory burst than macrophages and consequently are able to generate more reactive oxygen intermediates and reactive nitrogen intermediates. In addition, neutrophils express higher levels of defenses than macrophages do.

2.5.1.2.2. Eosinophils

Eosinophils, like neutrophils, are motile phagocytic cells that can migrate from the blood into the tissue spaces. Their phagocytic role is significantly less important than that of neutrophils, and it is thought that they play a role in the defense against parasitic organisms. The secreted contents of eosinophilic granules may damage the parasite membrane.

2.5.1.2.3. Basophils

Basophils are non phagocytic granulocytes that function by releasing pharmacologically active substances from their cytoplasmic granules. These substances play a major role in certain allergic responses.

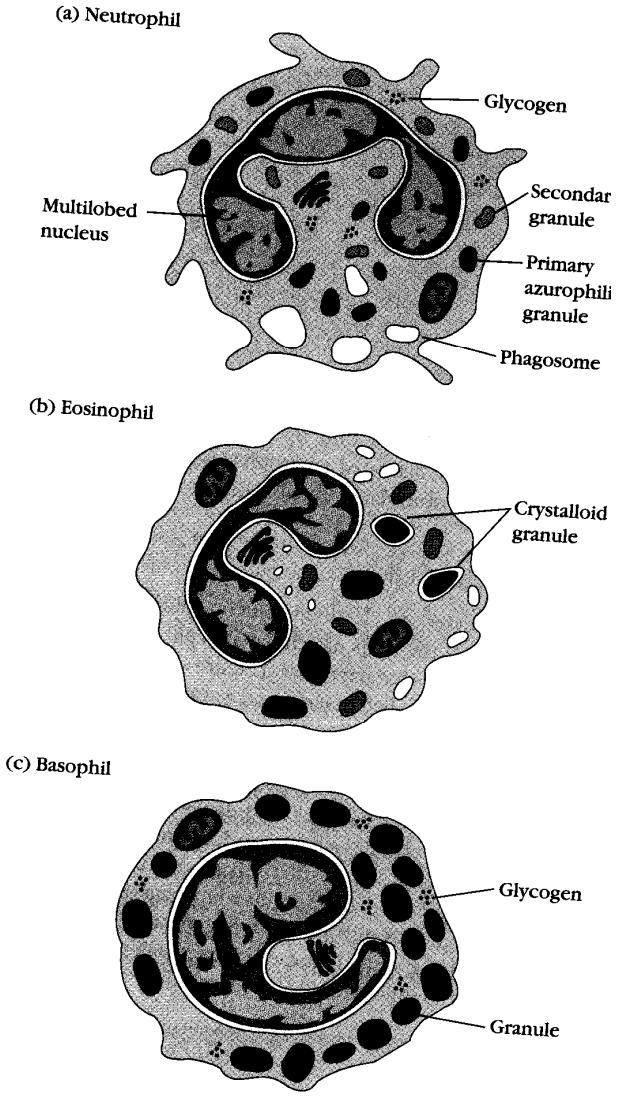

Fig. 2.1 Granulocytic cells

2.5.2. Mechanism of Phagocytosis

The process of phagocytosis occurs in following steps.

The signal: The entry of foreign particles such as microorganisms gives signal to the phagocytic cells in following ways: The signal to phagocytic cells is given by chemotactic substances derived from the complement system and lymphocyte derived factors. Microorganisms react with their Abs and then with C (complement). Some of

the components of C diffuse out from the center of reaction and establish a concentration gradient of chemotactic factors (C3a, C5), which attract microphages. The sensitised lymphocytes stimulated by specific Ag produce lymphokines such as macrophage chemotactic factor or macrophage activating factor that are chemotactic for monocytes and macrophages. The signal no macrophages is also given by products of the complement system.

Once the signal is noticed by phagocytic cell, it starts unidirectional movement towards an increasing concentration of chemotactic factors. The phagocytic cells move through the blood venules by a process called diapedesis.

Surface recognition: The engulfment of foreign particles largely depends upon surface properties of the particle to be phagocytosed, e.g., hydrophobicity and surface tension. Most of the nonpathogenic organisms are more hydrophobic than phagocytic cells, hence readily engulfed and destroyed. Organisms that are hydrophilic in nature, e.g., bacteria possessing hydrophilic capsule (pneumococci, *H. influenzae*), are difficult to engulf because of protective covering but the specific anticapsular Ab with or without complement neutralizes the charges on hydrophilic capsule, making it hydrophobic and increasing interfacial tension, so that it is readily engulfed by phagocytic cell.

Opsonisation: It is done by opsonins. Opsonins are of two types: Heat stable opsonins - Abs. Heat labile opsonins - complement components. Opsonins increase interfacial tension and hydrophobicity by reacting with particle to be phagocytosed hence it is readily phagocytosed.

Ingestion and digestion: Once the contact is made with foreign particle, engulfment starts with a deep invagination of cell membrane, which forms a thin layer around the particle. This fuses to form a pouch called phagosome. Phagosome penetrates deep into cytoplasm and fuses with lysosomal granules to form phagolysosome. The granules rupture, discharging their enzymatic contents into the vacuole and come into contact with the ingested particle. The ingested particle is slaughtered by a battery of mechanisms. Lysosome contains a variety of hydrolytic enzyme such as glucuronidases, lipases,

nucleases, peroxidases, phosphatases, lysozyme, phagocytin and other bactericidal substances, which quickly within 15 minutes kill most of the microorganisms

2.6. Mast Cells

Mast-cell precursors, which are formed in the bone marrow by hematopoiesis, are released into the blood as undifferentiated cells; they do not differentiate until they leave the blood and enter the tissues. Mast cells can be found in a wide variety of tissues, including the skin, connective tissues of various organs, and mucosal epithelial tissue of the respiratory, genitourinary, and digestive tracts. Like circulating basophils, these cells have large numbers of cytoplasmic granules that contain histamine and other pharmacologically active substances. Mast cells, together with blood basophils, play an important role in the development of allergies.

2.7. Dendritic cells

These are the Ag presenting cells like macrophages. They are derived from bone marrow and are different from the macrophages and T and B lymphocytes. They have little or no phagocytic activity. They are highly pleomorphic with a small central body and many long needle-like processes, resemble the dendrites of nerve cells They are present in peripheral blood and in the peripheral lymphoid organs, especially in the germinal centers of the spleen and lymph nodes. They are believed to play an important role in the presentation of antigens to T-cells during the primary immune response. Dendritic cells can be difficult to isolate because the conventional procedures for cell isolation tend to damage their long extensions. The development of isolation techniques that employ enzymes and gentler dispersion has facilitated isolation of these cells for study in vitro.

Most dendritic cells process and present antigen to TH cells. These cells can be classified by their location: Langerhans cells found in the epidermis (skin) and mucous membranes ; Interstitial dendritic cells, which populate most organs (e.g., heart, lungs, liver, kidney, gastrointestinal tract) ; Interdigitating dendritic cells present in T-cell areas of secondary lymphoid tissue and the thymic medulla ; Circulating dendritic cells include

those in the blood, which constitute 0.1% of the blood leukocytes, and those in the lymph (known as veiled cells)

The dendritic cells in different locations have different forms and functions. Despite their differences, all of these dendritic cells constitutively express high levels of both class II MHC molecules and members of the co-stimulatory B7 family. For this reason, they are more potent antigen-presenting cells than macrophages and B cells, both of which need to be activated before they can function as antigen-presenting cells (APCs). After capturing antigen in the tissues by phagocytosis or by endocytosis, dendritic cells migrate into the blood or lymph and circulate to various lymphoid organs, where they present the antigen to T lymphocytes.

Dendritic cells descend from hematopoietic stem cells through the myeloid lineage. The exact developmental path or paths taken by these cells is still under investigation. The major issue is whether dendritic cells belong from an entirely separate lineage. Two possible developmental pathways. In one pathway, a dendritic cell develops from a myeloid precursor in the bone marrow, then appears in the blood as an immature cell that completes its differentiation in the tissues. An alternative model proposes that a late mono-cytic-stage cell differentiates in the tissues to generate either a macrophage or dendritic cell. Some evidence suggests that mature macrophages and dendritic cells may interconvert, although this has yet to be confirmed. The morphologic and functional differences observed among Langerhans cells and interstitial, interdigitating, and circulating dendritic cells is though to reflect different maturational states of the cells and the different microenvironments in which they reside.

2.8. Summary

The cells participate in the immune response are white blood , or leukocytes. All leukocytes develop from a common multipotent hematopoeitic stem cell during hamatopoiesis . There are three types of lymphocytes : B cells, T cells and null cells. Naïve B and T lymphocytes, after interacting with antigen, enlarge into lymphoblasts that proliferate and eventually differentiate into effector and memory cells. Macrophages and neutrophils are specialized for the phagocyosis and degradation of antigens. Basophils

and mast cells are non phagocytic cells that release a variety of pharmacologically active substances and play an important roles in allergic reaction. Dendritic cells capture antigen. These cells along with macrophages and B cells, play an important role in T_H cell activation.

2.9. Model Questions

Write an essay on T cells and their role in Cell mediate immune reactions.

Give an account on types and subpopulations of B- lymphocytes and their functions.

Write an account on different types of mononuclear and granulocytic cells and their role in immune reactions.

Write short notes on

a) T- Lymphocytes
b) B- Lymphocytes
c) Basophils, Eosiophiles, Neutrophiles
d) Phagocytic cells
e) Phagocytosis
f) Dendritic cells

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PROF. K.V.MALLAIAH

LESSON NO. 3.

COMPONENTS OF IMMUNE SYSTEM – II
ORGANS

3.0. Objective : In this lesson, structure and functions of primary and secondary lymphoid organs and Mucosa associated lymphoid tissue, were described in detail.

3.1. Introduction

Lymphatic System

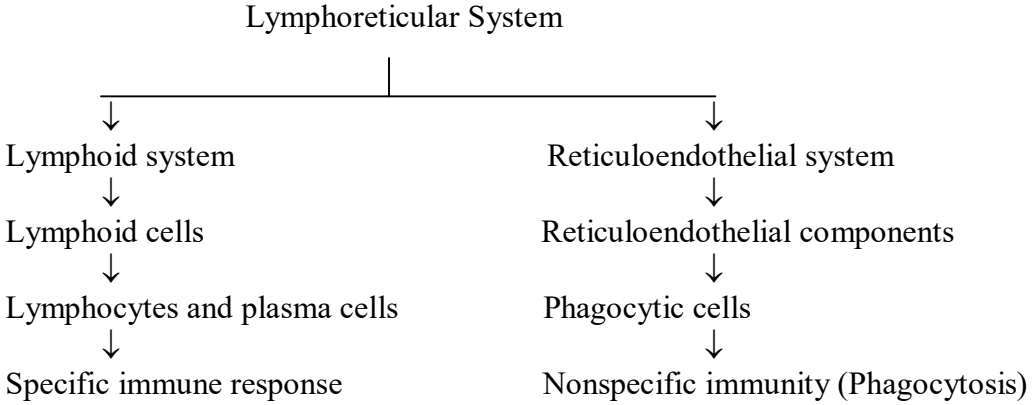
As blood circulates under pressure, its fluid component (Plasma) seeps through the thin wall of the capillaries into the surrounding tissue. Much of this fluid, called interstitial fluid, returns to the blood through the capillary membranes. The remainder of the interstitial fluid, now called lymph, flows from the spaces in connective tissue into a network of tiny open lymphatic capillaries and then into a series of progressively larger collecting vessels called lymphatic vessels

The largest lymphatic vessel, the thoracic duct, empties in to the left subclavian vein near the heart. In this way, the lymphatic system captures fluid lost from the blood and returns it to the blood, thus ensuring steady-state levels of fluid within the circulatory system. The heart does not pump the lymph through the lymphatic system; instead the flow of lymph is achieved as the lymph vessels are squeezed by movements of the body's muscles. A series of one-way valves along the lymphatic vessels ensures that lymph flows only in one direction.

When a foreign antigen gains entrance to the tissues, it is picked up by the lymphatic system (which drains all the tissues of the body) and is carried to various organized lymphoid tissues such as lymph nodes, which trap the foreign antigen. As lymph passes from the tissues to lymphatic vessels, it becomes progressively enriched in lymphocytes. Thus, the lymphatic system also serves as a means of transporting lymphocytes and antigen from the connective tissues to organized lymphoid tissues where the lymphocytes may interact with the trapped antigen and undergo activation.

The immune system comprises of a variety of organs, which are responsible for growth and development of immunocompetent cells. These are lymphoid organs and reticuloendothelial system (lymphoreticular system) (Table 3.1).

Table 3.1 Lymphoreticular system



The different categories of lymphocytes, plasma cells and macrophages (phagocytic cells) are the important cells, which participate in the different arms of the immune response to antigenic stimuli. Lymphocytes are the major immunological

effector cells. They arise from a precursor or stem cell, which originates in fetal life in the yolk sac and is found subsequently in the liver and bone marrow. The stem cells are further differentiated in two different directions. Some of them migrate to thymus where they are processed to acquire certain markers and functional characteristics that separate them from other lymphocyte population. These are thymus derived or T-lymphocytes, which play an important role in cell mediated immunity. Other stem cells are processed in the bone marrow of the bursa of fabricius (in chicken) to acquire surface markers that differentiate them from T-lymphocytes. These are B-lymphocytes, which play an important role in humoral or antibody mediated immunity.

Both T and B cells, after maturation, migrate to spleen, lymph nodes and other organs where they initiate and participate in immune response to antigens.

The lymphoid system consists of: Lymphoid organs, Lymphoid cells (lymphocytes and plasma cells).The lymphoid organs are classified into the central (primary) and the peripheral (secondary) lymphoid organs based on different functions they perform.

3.2. Central (Primary) Lymphoid Organs

These are the lymphoid organs in which proliferation and differentiation of lymphocytes takes place without antigenic stimulation (antigen independent maturation of lymphocytes). The primary lymphoid organs include thymus, bursa of fabricius, bone marrow and intestinal epithelium.

3.2.1. Thymus: (Fig.3.1) It is a lymphoepithelial bilobed structure located behind the upper part of the sternum (in the anterior mediastinum). It is derived from the third and fourth pharyngeal pouches and differentiated from these pouches at about the sixth week of fetal life. It acquires characteristic lymphoid appearance by the third month of gestation. It increases in size during foetal development, reaches its maximum at birth and gradually decreases in size with age and finally atrophies. It is capsulated. The septa

arising from the capsule divide the gland into lobules, which are differentiated into an outer cortex and an inner medulla. The cortex is composed mainly of epithelial cells and lymphocytes. The precursors of lymphocytes (stem cells---immature cells) from yolk sac, foetal liver and bone marrow reach the thymus and mature in the cortex, acquire surface characteristics of T-lymphocytes and then migrate into medulla. In the medulla, the lymphocytes complete their maturation process and exit into the blood as matured T-cells capable or responding to antigenic stimuli and seeded into the secondary lymphoid organs. Mature thymocytes in thymus are about 5-10% of the total population.

Functions of thymus: It is the center for development and function of the immune system, however, it does not participate in immune reaction. It is the major site for lymphocyte proliferation and production of T-lymphocytes. In the thymus, lymphocytes acquire new surface antigens. The thymus confers immunological competence on the lymphocyte. Pre thymic lymphocytes are not immunologically competent. In the thymus, they are educated by hormone like humoral factors--thymosin, thymopoietin, etc., produced by thymic epithelial cells, so that they become capable of mounting CMI.

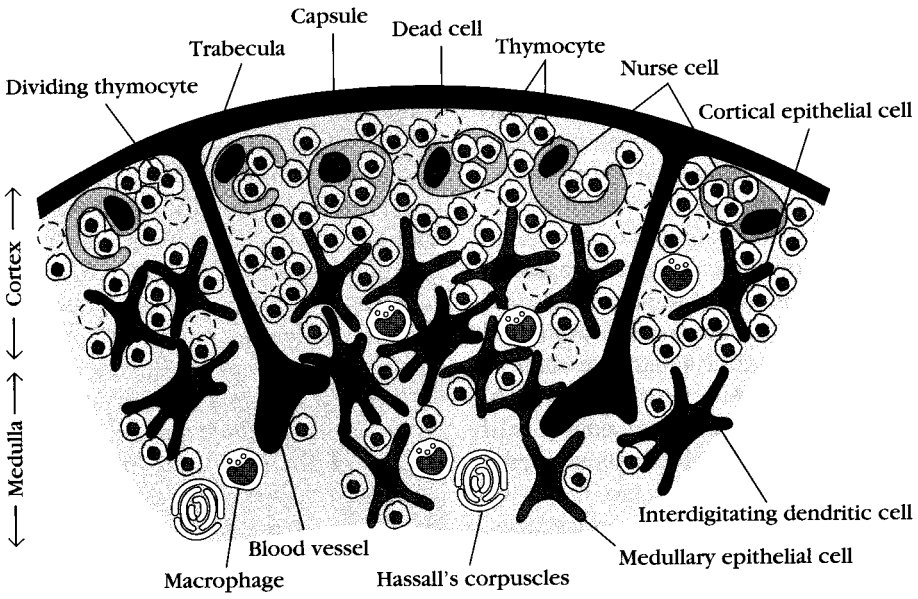


Fig 3.1. Diagrammatic cross section of a portion of the thymus

3.2.2. Bursa of fabricius: It is a lymphoepithelial organ located near the cloaca in chickens. It is similar to thymus and developed from lymphocytic infiltration of an epithelial out pouching from the cloaca. It contains lymphoid follicles, which are divided into cortex and medulla and composed of immunoglobulin producing B-cells. Precursors of B-cells (stem cells) from the yolk sac, foetal liver and bone marrow reach the bursa and mature into immunocompetent bursal lymphocytes or B-cells, which exit through the peripheral blood and become seeded into the secondary lymphoid organs.

Functions: It is responsible for the development of immunocompetent B-cells making humoral antibodies. In mammals, equivalent of bursa has not yet been identified. The maturation of precursors of B-cell into functionally mature B-lymphocyte occurs in bone marrow and gut associated lymphoid tissues.

3.2.3. Intestinal epithelium: Thymus is the main site for production of T-cells. Recently, it has found that the intestinal epithelium is also a major site for development of T-cells. These T-cells are known as thymus independent T-cells.

3.3. Peripheral (secondary) Lymphoid Organs

These are the organs, which receive and maintain functional lymphocytes. The lymphocytes educated by central lymphoid organs are seeded into peripheral lymphoid organs where they initiate and participate in immune response to antigenic stimuli. The peripheral lymphoid organs include lymph nodes, spleen and mucosa associated lymphoid tissue.

Mechanism : Various types of organized lymphoid tissues and diffuse collections of lymphocytes and macrophages are present in these organs. The lymphoid tissue is organized into structures called lymphoid follicles, which consist of aggregates of lymphoid and non lymphoid cells surrounded by a network of draining lymphatic capillaries. Until it is activated by antigen, a lymphoid follicle-called a primary follicle-comprises a network of follicular dendritic cells and small resting B cells. After an antigenic challenge, a primary follicle becomes a larger secondary follicle-a ring of

concentrically packed B lymphocytes surrounding a center (the germinal center) in which one finds a focus of proliferating B lymphocytes and an area that contains non dividing B cells, and some helper T cells interspersed with macrophages and follicular dendritic cells.

Most antigen-activated B cells divide and differentiate into antibody-producing plasma cells in lymphoid follicles, but only a few B cells in the antigen-activated population find their way into germinal centers. Those that do enter a germinal center undergo one or more rounds of cell division, during which the genes that encode their antibodies mutate at an unusually high rate. Following the period of division and mutation, there is a rigorous selection process in which more than 90% of these B cells die by apoptosis. In general, those B cells producing antibodies that bind antigen more strongly have a much better chance of surviving than do their weaker companions. The small number of B cells that survive the germinal center's rigorous selection differentiate into plasma cells or memory cells and emerge.

3.3.1. Lymph nodes: These are small, round or oval shaped organs found in various parts of the body. They are generally located at major junctions of the network of lymphatic channels that connect to the thoracic duct, which passes lymphocytes and lymph to the large vein connected to the heart. They are differentiated into outer cortex and inner medulla. The cortex is further subdivided into the external cortex located just below the capsule and a deep cortex also known as the paracortical area. In the external cortex, there is accumulation of lymphocytes in regions called follicles (primary lymphoid follicles) within which there is development of germinal centers (secondary follicles) following antigenic stimulation. The follicles contain small lymphocytes and dendritic macrophages, which are responsible for trapping and processing of antigen. In the medulla, lymphocytes are arranged as elongated branching bands (medullary cords). In activated lymph nodes, most of them are plasma cells secreting antibodies. The cortical follicles and medullary cords contain B-lymphocytes (bursa dependent area) while the T-cells are occupied in paracortical area (thymus dependent area) (Fig. 3.2).

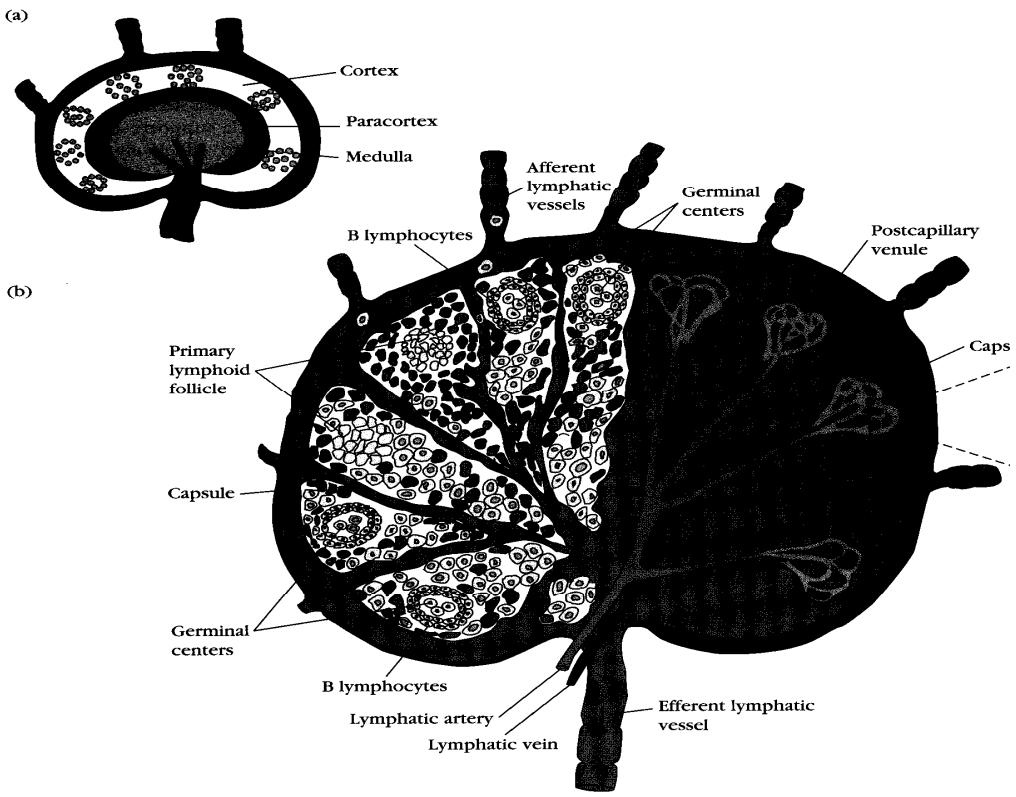


Fig 3.2 Structure of a lymph node. (a) The three layers of a lymph node (b) various regions within the lymph node.

Functions of lymph node: They act as filters for lymph draining body tissues including foreign antigens. They act as junction between circulatory system and lymphatic system. Provide a site for phagocytosis and antibody production. Support the development of lymphocytes. Allow recirculation of lymphocytes.

3.3.2. Spleen: It is the largest lymphovascular organ about 250g in weight in adult man. The architecture is similar, but not identical to that of lymph node. It has two segregated area; the red pulp and while pulp, separated by marginal zone. The white pulp is rich in lymphoid tissue while the red pulp is abundant in sinuses and contains large quantities of RBCs. The white pulp is located mainly around small arteries and the peri arterial lymphatic sheath is composed primarily of T-lymphocytes and is called the thymus

dependent area. The external lymphoid area surrounding the peri arterial lymphatic sheath is a B-dependent area (peri-follicular region, germinal center and mantle layer). Approximately 30-40% of the cells in the spleen are T-cells and 50% of the cells are B-cells. The periarterial lymphoid collections in the white pulp are called Malpighian corpuscles or follicles. Following antigenic stimulation, germinal centers are produced in the white pulp that are composed of large numbers of rapidly dividing cells, which differentiate into plasma B-cells and plasma cells and replace T-cells in the periarterial region (Fig. 3.3).

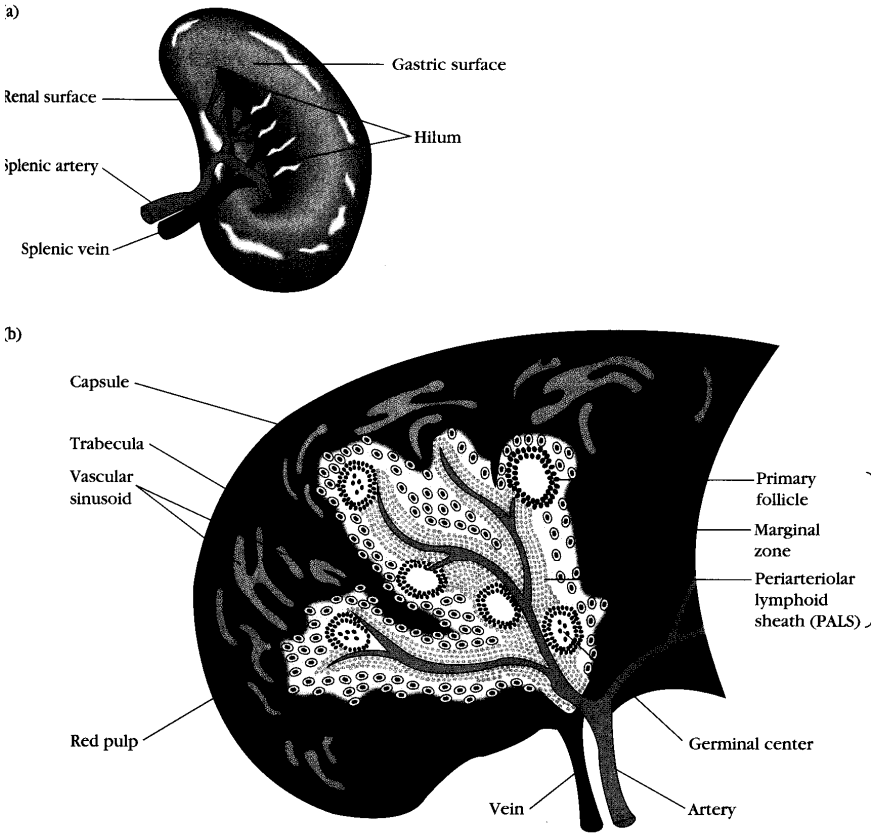


Fig.3.3. Structure of the spleen (a) The spleen whole organ (b) diagrammatic cross section of the part of the spleen

Functions of spleen: It is the sole lymphatic organ specialized to filter blood. Thus, it is a systemic filter to trap or concentrate circulating blood born particles. It is a major site

for antibody synthesis against blood borne particles including microorganisms. Its immunological role is primarily directed against blood borne pathogens.

3.3.3. Mucosa associated lymphoid tissue (MALT):

Lymph nodes and the spleen are the most highly organized of the secondary lymphoid organs; they comprise not only lymphoid follicles, but additional distinct regions of T-cell and B-cell activity, and they are surrounded by a fibrous capsule. Less-organized lymphoid tissue, collectively called mucosal-associated lymphoid tissue, (MALT), is found in various body sites. Potentially important collections of lymphocytes, mainly producing IgA are present throughout the mucosal lining of alimentary, respiratory, genitourinary and other surfaces. The MALT structure contains mixture of B-cells, T-cells as well as phagocytic cells. Secretory IgA is the main immunoglobulin produced by MALT, IgG, IgM and IgE are also produced locally.

MALT includes peyer's patches (in the small intestine), the tonsils, and the appendix, as well as numerous lymphoid follicles within the lamina propria of the intestines and in the mucous membranes living the upper airways, bronchi, and genital tract. However, these different lymphoid tissues can also be categorized according to their places of occurrence in the body. Like, lymphoid tissues of the gut are known as gut associated lymphoid tissue (GALT) and those in the respiratory tract are called the bronchus associated lymphoid tissue (BALT). The main GALT structures in man are: Tonsils (lingual, palatine and pharyngeal); Appendix (at the junction of small and large intestine) (Fig. 3.4.); Peyer's patches of the intestine; and Lamina propria of the intestine. BALT includes the tissues in the upper airways, bronchi, and genital tract.

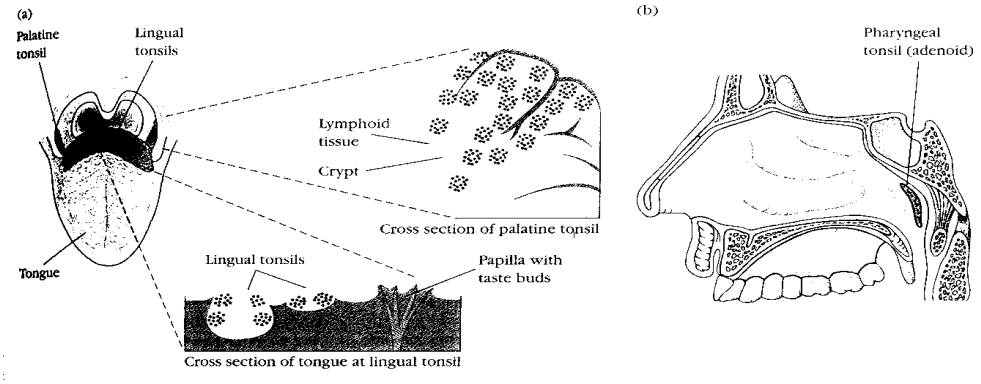


Fig. 3.4. Three types of tonsils. a) The position and internal features of the palatine and lingual tonsils b) a view of the position of the nasopharyngeal tonsils.

The mucous membranes lining the digestive, respiratory, and urino-genital systems have a combined surface area of about 400 m^2 and are the major sites of entry for most pathogens. These vulnerable membrane surfaces are defended by a group of organized lymphoid tissues known collectively as mucosal-associated lymphoid tissue (MALT). Structurally, these tissues range from loose, barely organized clusters of lymphoid cells in the lamina propria of intestinal villi to well-organized structures such as the familiar tonsils and appendix, as well as peyer's patches, which are found within the submucosal layer of the intestinal lining. The functional importance of MALT in the body's producing plasma cells, whose number far exceeds that of plasma cells in the spleen, lymph nodes, and bone marrow combined.

The tonsils are found in three locations: lingual at the base of the tongue; palatine at the sides of the back of the mouth; and nasopharyngeal (adenoids) in the roof of the nasopharynx. All three tonsil groups are nodular structures consisting of a meshwork of reticular cells and pulp surrounds the sinusoids. The white pulp forms a sleeve, the periarteriolar lymphoid sheath (PALS) around the arterioles; this sheath contains numerous T cells. Closely associated with the PALS is the marginal zone, an area rich in B cells that contains lymphoid follicles that can develop into secondary follicles containing germinal centers. Fibers interspersed with lymphocytes, macrophages, granulocytes, and mast cells. The B cells are organized into follicles and germinal

centers; the latter are surrounded by regions showing T-cell activity. The tonsils defend against antigens entering though the nasal and oral epithelial routs.

The best studied of the mucous membranes is the one that lines the gastrointestinal tract. Lymphoid cells are found in various regions within this tissue. The outer mucosal epithelial layer contains so-called intraepithelial lymphocytes (IELs). The majority of these lymphocytes are T cell that express unusual receptors (γδ T-cell receptors, or γδ TCRs), which exhibit limited diversity for antigen. Although this population of T cells is well situated to encounter antigens that enter through the intestinal mucous epithelium, their actual function remains largely unknown. The lamina propria, which lies under the epithelial layer, contains large numbers of B cells, plasma cells, activated TH cells, and macrophages in loose clusters. Histological sections have revealed more than 15,000 lymphoid follicles within the intestinal lamina propria of a healthy child. The submucosal layer beneath the lamina propria contains peyer's patches, nodules of 30-40 lymphoid follicles. Like lymphoid follicles in other sites, those that compose peyer's patches can develop into secondary follicles with germinal centers.

The epithelial cell of mucous membranes play an important role in promoting the immune response by delivering small samples of foreign antigen from the lumina of the respiratory, digestive, and urino-genital tracts to the underlying mucosal-associated lymphoid tissue. This antigen transport is carried out by specialized cells, called M cells. The structure of the M cell is striking: these are flattened epithelial cells lacking the microvilli that characterize the rest of the mucous epithelium. In addition, M cells have a deep invagination, or pocket, in the basolateral plasma membrane; this pocket is filled with a cluster of B cells, T cells, and macrophages. Luminal antigens are endocytosed into vesicles that are transported from the luminal membrane to the underlying pocket membrane. The vesicles then fuse with the pocket membrane, delivering the potentially response-activating antigens to the clusters of lymphocytes contained within the pocket.

M cells are located in so-called inductive sites-small regions of a mucous membrane that lie over organized lymphoid follicles. Antigens transported across the mucous membrane by M cells can activate B cells within these lymphoid follicles. The activated B cells differentiate into plasma cells, which leave the follicles and secrete the IgA into the lumen, where they can interact with antigens.

Mucous membranes are an effective barrier to the entrance of most pathogens, which thereby contributes to nonspecific immunity. One reason for this is that the mucosal epithelial cells are cemented to one another by tight junctions that make it difficult for pathogens to penetrate. Interestingly, some enteric pathogens, including both bacteria and viruses, have exploited the M cell as an entry route through the mucous-membrane barrier. In some cases, the pathogen is internalized by the M cell and transported into the pocket. In other cases, the pathogen binds to the M cell and disrupts the cell, thus allowing entry of the pathogen. Among the pathogens that use M cells in these ways are several invasive *Salmonella* species, *Vibrio cholerae*, and the *polio* virus.

Functions of MALT: Local immunity against pathogens invading local tissue.

The T and B lymphocytes are two groups of non-phagocytic, morphologically indistinguishable, functionally different cells but having certain common features: They re-circulate throughout the body from blood to tissue and again into circulation. There is constant traffic of lymphocytes through the blood, lymph, lymphatic organs and tissues, so that when an antigen enters into any part of the body, lymphocytes of appropriate specificity would reach the site and mount an immune response. Re-circulating lymphocytes can be recruited by the lymphoid tissues whenever necessary. Majority of the re-circulating cells are T-cells. B-cells tend to be more sessile. Each lymphocyte possesses specific receptor on its surface for recognition of particular antigen. This confers specificity. When stimulated by specific Ag, the lymphocytes undergo clonal proliferation. B-cells are transformed into plasma cells which synthesize antibodies while T-cells produce lymphokines and induce CMI. They show a property of memory giving rise to a faster and powerful immune response on re-challenge with same antigen.

3.4. Summary

The primary lymphoid organs provide site where lymphocytes mature and become antigenically committed. T lymphocytes mature within the thymus and B lymphocytes arise and mature within the bone marrow of humans. Secondary lymphoid organs capture antigens and provides sites where lymphocytes become activated by interaction with

antigens. Lymph nodes trap antigen from lymph, spleen traps blood borne antigens, intestinal associated lymphoid tissues interact with antigens that enter the body from the gasterointestinal trac. Lymphocytes present throughout the mucosal lining of alimentary, respiratory, genitourinary and other surfaces helps in local immunity against pathogens invading local tissue.

3.5 Model Questions

Write an essay on structure and functions of primary lymphoid organs.

Write in detail about structure and functions of secondary lymphoid organs.

Give an account on various tissues of MALT and its functions.

Write short notes on

a) Lymph nodes
b) Thymus
c) Bone marrow
d) Spleen
e) MALT

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LESSON NO. 4.

MAJOR HISTOCOMPATIBILITY COMPLEX AND SOLUBLE MEDIATORS

4.0. Objectives : This lesson mainly deals with structure and overall functions of Major histocompatibilty molecules and role of different soluble mediators in immune responses.

4.1. Introduction

The major histocompatibility complex (MHC) is a region on chromosome consisting of a closely linked cluster of genes that code for a number of cell surface and plasma protein antigens. The MHC in mammals also consists of a region where the histocompatibility linked immune response genes (IR) are located. This chromosomal segment controls: synthesis of transplantation antigens and graft rejection ; immune response to infection ; and susceptibility to the development of immunologically

mediated diseases. The two MHC systems that have been most extensively studied are: The H2 antigen system in the mouse. The HLA (Human Leukocyte Antigen) system in man. The major antigens determining histocompatibility in human beings are alloantigens found on the surface of leucocytes (hence are known as HLA Ags) and the MHC system is known as the HLA system.

4.1.1. HLA complex:

Originally the concept of histocompatibility arise from transplantation rejections by the body. In tissue transplantation rejections, products of the major histocompatibility complex genes and T cells play an important role. Every vertebrate species has MHC genes and products but most detailed information is known about the human and mouse systems.

The concept that the rejection of foreign tissue is the result of an immune response to cell-surface molecules now called histocompatibility antigens. The major histocompatibility complex is a collection of genes arrayed within a long continuous stretch of DNA on chromosome 6 in humans and on chromosome 17 in mice. The MHC is referred to as the HLA complex in humans and as the H-2 complex in mice The MHC genes are organized in to regions encoding three classes of molecules.

Class I MHC genes encode glycoproteins expressed on the surface of nearly all nucleated cells; the major function of the class I gene products is presentation of peptide antigens to Tc cells.

Class II MHC genes encode glycoproteins expressed primarily on antigen presenting cells (macrophages, dendritic cells and B cells) where they present processed antigenic peptides to T_H cells.

Class III MHC genes encode in addition to other products, various secreted proteins that have immune functions, including components of the complex system and molecules

involved in inflammation. E.g. C2 and C4 of the classical pathway, properdin factor of the alternative pathway and tumour necrosis factor **α** and **β (TNF)**.

The major function of MHC is antigen presentation and processing. Pathogens such as bacteria and viruses can penetrate and infect the cells of the body and T cells mount an immune response against the cells harboring the invading organism. The foreign antigens that trigger an immune response are of two types. First, when the pathogens enter the body, the antigens derived from these organisms are called exogenous antigens. Secondly, the antigens made inside the body. For example the new viral proteins synthesized in the virus infected cells. If all foreign material that gets into the body were totally ingested, digested by phagocytic cell, their would be no need of stimulus for an immune response. This may in fact be a common occurrence. Nevertheless, some antigens must persist to stimulate antigen-sensitive cells and initiate an immune response. These cells are called antigen presenting cells. In side these cells, the proteins are broken-down in to smaller fragments, peptides. Some of these peptides associate inside the cell with MHC class I or class II molecules, and the resulted peptide-MHC complex moves to the surface of the cell where it can be recognize by a T cell with an appropriate receptor. The events involved in the generation of peptides from proteins inside cells, the binding of peptides to MHC molecules and the display of peptide-MHC complexes at the cell surfaces for the T cell recognition are known collectively as **antigen processing and presentation**.

MHC class I and II molecules have different functions in T cell responses. The function of MHC class I molecules is to present the peptides derived from protein antigens to CD8+ T cells. By contrast, the function of MHC class II molecules is to present peptides to CD4+ T cells. Exogenous antigens are those antigens taken in to cells, normally by endocytosis or phagocytosis. Exogenous antigens can be derived from pathogens (example bacteria and viruses) and from foreign proteins (example ovalbumin and sheep red blood cells) that do not injure the host but activate an immune response. Responses to exogenous antigens taken up by APCs – dendritic cells, B cells or macrophages.

Endogenous antigens are synthesized inside a cell, typically, they are derived from pathogens that have infected the cells. Processing of endogenous antigens occurs in the cytoplasm rather than in the acid vesicles in which exogenous antigens are processed. The major mechanism for generating peptide fragments in the cytoplasm is via a giant protein complex known as the proteasome. This cleaves into peptides about 15 amino acids in length. Cytosolic enzymes (aminopeptidases) remove even more amino acids from the peptides.

4.1.2. Histocompatibility molecules (Antigens)

a) **Class I molecules (antigens) (Fig.4.1.) :** The class I antigen is - a transmembrane (**α**-chain) glycoprotein having about 350 amino acids. Molecular weight is 44 kD. Non-covalently associated with **β**-2 macroglobulin (**β**-chain) having 100 amino acids of an molecular weight of12kD. The **β** chain (**β**-2 macroglobulin) is a plasma protein, encoded by a gene outside the MHC and located on chromosome -15. The **α**-chain is encoded by three structural genes in the HLA- A, B and C regions and its products are the HLA - A, B and C antigens. The MHC class I products (antigens) are found on the surface of most nucleated cells and most abundantly on lymphoid cells. They are absent on sperm and trophoblastic cells. They are detected by their reactivity with human or mouse alloantisera.

Other important functions of class I antigens: Essential for immune T-cell recognition of specific target antigens. Lymphocytes can only bind to antigens that are associated with these molecules. Class I antigens are strong transplant antigens responsible for graft rejection and elimination of virus infected cells (cell mediated cytolysis). They may function as components of hormone receptors. The CD-8 cells are specific for MHC-class I antigens.

b) Class II molecules (antigens) (Fig.4.2.) : It is an heterodimer consisting of two non-covalently bound glycoprotein chains called α and β chains of about 34 kD and 29kD molecular weight, respectively. They are anchored in the cell surface membrane. Each chain has two extracellular domains, transmembrane portion and short intracytoplasmic region. The proximal domain is the constant region and the distal domain is variable α-1 and β-1 (the two distal domains) constitute the Ag-binding site for recognition by CD-4 lymphocytes. The class II antigens are distributed on limited cell types and are found on macrophages, dendritic cells, activated T-lymphocytes (CD-4) and B-lymphocytes. These antigens are encoded by genes of HLA - DR, DQ and DP regions.

Additional functions of class II antigens: They play a major role in: Graft versus host response. Immune responsiveness. Immune suppression. Cellular recognition. Cellular interactions. Mixed lymphocyte reaction. The functions of domains are recognition of Ig, cell surface Ags (Thy 1, T-cell surface antigens, T4 and T8) and the T-cell antigen receptor.

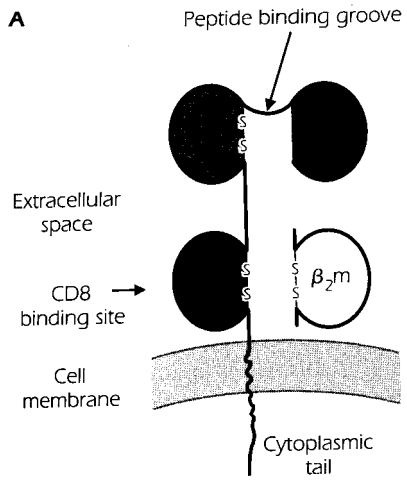


Fig 4.1. Diagram of the structure of an MHC class I molecule

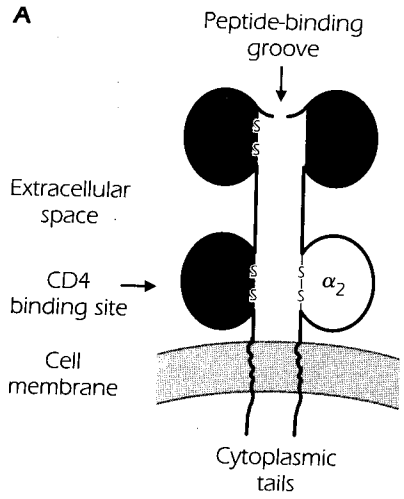


Fig 4.2. Diagram of the structure of an MHC class II molecule

c) Class III antigens: These are heterogenous molecules. Include complement components C2, C4 and factor B (components responsible for the formation of C3 convertase). They also include heat shock proteins and tumour necrosis factor. These molecules are encoded by class III MHC genes.

4.1.3. Overall function of MHC: MHC class I and class II genes and their products provide a system for intracellular communication. MHC antigens are essential for recognition of antigen by T-cells. T-cells recognize antigen when it is presented in association with class I and class II histocompatibility antigens. T-helper cells with CD4 molecules recognize foreign antigen in association with class II antigen whereas cytotoxic T-cells with CD8 molecules are restricted by Class-I antigens. MHC class III products are the important components of complement system and are responsible for various cellular events.

4.2. SOLUBLE MEDIATORS

4.2.1. Cytokines and Lymphokines

As a result of appropriate stimulation, cells of the immune system secrete a wide variety of proteins that mediate signaling between cell and cell to control the immune

responses. The generic term for these regulatory proteins secreted by a cells is called **Cytokines** .

Different terminology is used for cytokines based on the cells which secrete them and also on their functions.

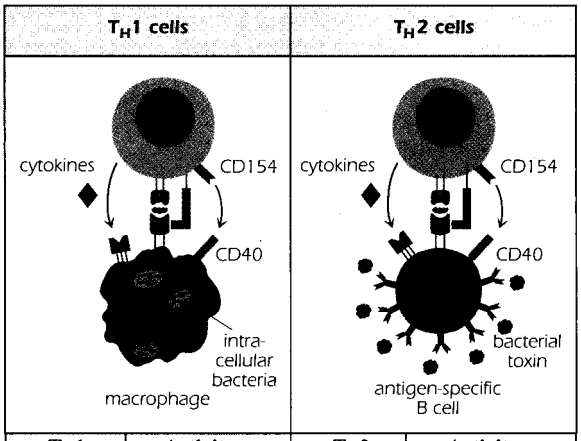
Cytokines secreted by lymphocytes are called **Lymphokines** and those produced by monocytes or macrophages are called Monokines**.** Most lymphokines exhibit multiple biological effects and the same effect may be caused by different lymphokines. The term interleukin is introduced for those products of leukocytes, which exert regulatory influence on other leukocyte cells. The lymphokines, monokines, interferons, growth factors and other factors have similar effects. Therefore, they are grouped as cytokines.

Cytokines are low molecular weight antigen-non specific proteins that mediate cellular interactions involving immuno, inflammatory and hematopoietic systems. Cytokines are short lived and may act locally either on the same cell that secreted it (autocrine) or on other cells (paracrine). Like hormones, they may act systemically (endcrine). Cytokine have a vide variety of functional activities as illustrated their ability to 1) Regulate specific immune responses 2) facilitate innate immuno responses, 3) activate inflammatory responses, 4) affect leukocyte movement, and 5) stimulate hematopoiesis.

Lymphokines are biologically active substances released by activated T-lymphocytes. These are regulatory proteins (mol wt. 20,000-80,000) whose main function is regulation of immune response and growth and function of cells of reticuloendothelial system. They have several biological activities.

Cytokines are mainly produced by macrophages, while lymphokines are produced mainly by T cells. Macrophages produce four major classes of cytokines, namely IL-1, IL-6, IL-12 and TNF-α. T cells produce many different lymphokines (Fig.4.3.) that form two major groups- those produced by Th1 cells and those produced by Th2 cells. In general, the Th1 derived lymphokines tend to have biological activities that counteract the

activities of the Th2 derived lymphokines. Lymphokines produced by Th1 cells are IL-2, INF-γ, and lymphotoxin (Tumor Necrosis Factor-β) and those produced by Th2 cells are IL-4, IL-5, IL-9, IL-10 and IL-13.



T_H1 Cytokines	Activity	T_H2 Cytokines	Activity
IFN-γ	Activates macrophages; Regulates cells	IL-4	Activates B cells
GM-CSF	Activates macrophages	IL-5	Activates B cells
TNF-α	Activates macrophages	IL-10	Inhibits macrophages
IL-3	Involved in hematopoiesis	IL-3	Involved in hematopoiesis
TNF-β	Involved in inflammatory responses	TGF-β	Inhibits monocyte activation

Fig. 4.3. Cytokines produced by Th 1 and Th 2 cells

4.2.3. Types of Cytokines

4.2.3.1. Cytokines affecting lymphocytes

The biological activities of different cytokines are as follows

Interleukin-1 (IL-1)

It is a polypeptide, a monokine released by macrophages and other Ag presenting cells. It is heat labile, retains its activity up to 56°C and pH 3-11. It occurs in two molecular forms as IL-1 α and IL-1β, which have exactly same function. Its production is stimulated by Ags, toxins, injury and inflammatory processes. Its production is inhibited

by cyclosporine - A, corticosteroids and prostaglandins. Immunological effects of IL-1 are - It stimulates activation of T-helper (Th) cells for the production of IL-2 and other lymphokines. Stimulation of B-cell proliferation and Ab synthesis; granulocytes (neutrophil chemotaxis) and phagocytosis. It mediates a wide range of metabolic, physiological, inflammatory, and haematological effects by acting on bone marrow, epithelial and synovial cells, fibroblasts, hepatocytes, vascular endothelium, and other targets. It is an endogenous pyrogen that induces fever by acting on hypothalamus. Along with tumour necrosis factor (TNF), it induces haematological changes in spetic shock and also enhances initial meningeal inflammation in bacterial meningitis. It has a beneficial effect in severe infections in immunocompromised hosts.

Interleukin - 2 (IL-2)

It is a cytokine produced by activated T-cells. It is a T-cell growth factor that induces proliferation of T-cell by binding to IL-2 receptor on the cells. It is a powerful modulator of the immune response. It promotes growth and differentiation of T and B cells. It stimulates cytotoxic T-cells and NK-cells. It converts large granular lymphocytes into lymphokine activated killer (LAK), cells which can destroy NK-resistant tumour cells. This property can be used in the treatment of certain types of cancers. Stimulates secretion of other lymphokines.

Interleukin - 3 (IL-3)

It is produced by T-cells. It acts as a growth factor for bone marrow stem cells. It stimulates multilineage haematopoiesis and therefore also known as the multicolony stimulating factor (multi CSF).

Interleukin - 4 (IL-4)

It is produced by T-helper (Th) cells. It activates resting B-cells and acts a B-cell differentiating factor. It also acts as a growth factor for T-cells and mast cells. It

enhances the activity of cytotoxic T-cells. It increase synthesis of IgG-1 and IgE and may play a role in atopic hypersensitivity.

Interleukin - 5 (IL-5)

Produced by T-helper (Th2) cells. It causes proliferation of activated B-cells and eosinophils. Stimulates production of IgA and IgM. In conjunction with IL-2, IL5 induces cytotoxic T-cell activity

Interleukin - 6 (IL-6)

Produced by stimulated T and B cells, macrophages and fibroblasts. It promotes terminal differentiation of B-cells into Ab producing plasma cells and encourages IgG production. It has properties in common with IL-1 can act on many cell types. Thus, it acts on B cells as a cofactor with IL-1 in IgM synthesis and IL-5 in Ig A synthesis. It induces formation of IL-2 receptors on T-cells. It has a stimulatory effect on hepatocytes, nerve cells and haematopoietic cells. It acts as an inflammatory response mediator in host defense against infections.

Interleukin- 7 (IL-7)

It is derived from bone marrow and thymic stromal cell. It induced proliferation of pre-B cells, thymocytes and T cells and probably controls the lymphoid stem cells.

Interleukin- 9 (IL-9)

It is a single chain glycoprotein secreted by activated Th 2 cells. It induces antigen independent growth of certain helper T cells clones but has no effect on Cytotoxic t cell clones. It enhances the proliferative response of bone marrow derived mast cells to IL-3. It also potentiates the effect of IL-4 on B cell Ig E production.

Interleukin- 10 (IL-10)

In mice IL-10 is immunosupressive glycoprotein secreted by Th2 cells by some B-cells and by activated macrophages . It was originally called cytokine synthesis inhibiting factor (CSIF). Since it appeared to down regulate cytokine production by Th1 cells in mice.

Interleukin- 11 (IL-11)

It is a protein secreted by bone marrow stroma cells (fibroblasts) . It acts as a growth factor on certain B-cell lines in association with Il-6. It also stimulates megakaryocyte colony formation in association with IL-3 and so may have a role in stimulating platelet production.

Interleukin- 12 (IL-12)

It is produced by all the antigen processing cells (macrophages, dendritic cells, and B-cells) , acts on Th1 cells and NK cells. It promotes Th1 cell differentiation from the Th0 stage. It is the co stimulator of Th1 activity, inducing those cells to secrete IL-2 and IFN-γ and express IL-2R. It also inhibits some Th2 cell functions such as Ig E formation.

Interleukin- 13 (IL-13)

It is a protein produced by activated Th2 cells . Its production is induced by ligation of CD 28 on the T cell surface with a B7 or related molecule on the surface of an activated antigen presenting cell. It effects similar to those of IL-4.

Interleukin- 14 (IL-14)

A B-cell growth factor produced by T-cells and some malignant B-cells has been called interleukin 14. It is a secreted protein that induces B-cell proliferation, inhibits immunoglobulin secretion and selectively expands some B-cell sub-populations.

Interleukin- 15 (IL-15)

It is produced by wide variety of cells especially peripheral blood mononeuclear cells and epithelial and fibroblast cell lines. It has biological activities that are similar to IL-2 and acts as a T-cell growth factor. It enhances proliferation of both helper (CD4) and cytotoxic (CD8) T-cell populations.

Mitogenic or blastogenic factor (MF or BF)

Released by sensitised T-cells stimulated by specific antigen. It induces non-specific blast transformation of normal unsensitised T-lymphocytes. Along with transfer factor, it may be important in augmenting or amplifying the cell mediated response by recruiting uncommitted lymphocytes.

Transfer factor (TF)

An extract from specific antigen sensitised lymphocytes that mediates passive transfer of CMI is known as transfer factor. Both dialyzable and non-dialyzable transfer factors have been identified. The dialyzable transfer factor has low molecular weight (2000-4000). It is stable at 37^{o}C, at -20^{o}C and in the lyophilized from at 4^{o}C. It is inactivated at 56^{o}C in 30 minutes. It is resistant to treatment with DNAase, RNAase and trypsin and freeze thawing. Chemically, it is a polypeptide-polynucleotide. It is immunologically specific, i.e. it confers reactivity towards the antigen responsible for its generation. It is highly potent, an extract from 0.1ml of packed leucocytes is sufficient to

transfer CMI. Applications: It is useful in immunocompromised individuals to restore specific CMI, e.g., in T-cell deficiency (Wiskott-Aldrich syndrome, Nezelof syndrome, DiGeorge syndrome). Disseminated infections associated with deficient CMI (tuberculosis, lepromatous leprosy, mucocutaneous candidiasis, etc.). Cancer--melanoma, sarcoma, etc.

4.2.3.2. Cytokines affecting macrophages

Antigenically stimulated T-lymphocytes produce certain biologically active soluble proteins (lymphokines), which recruit, activate, and regulate effector cells with the potential to fight with the infecting agent.

The biological activities of lymphokines are as follows.

Macrophage chemotactic factor (MCF): It is chemotactic for mononuclear phagocytes. It causes accumulation of these cells at the site of Ag-mediated lymphokine release.

Migration inhibiting factor (MIF): It inhibits migration of phagocytic cells and localizes circulating and tissue monocytes at the site of infection. Once attracted or accumulated, cells are discouraged from leaving the site by this factor.

Interferon -γ (IFN-γ): It is also know as macrophage activating factor (MAF). It possesses powerful macrophage activating molecules. It produces significant morphological changes. It increases content of lysosomal enzymes and their activities, so that the macrophage kills ingested intracellular foreign particles. It also causes augmentation of neutrophil and monocyte functions and has antitumour activity.

Interferon-α (IFN-α): It is produced by leucocytes. It has antiviral activity.

Interferon-β (IFN-β): It is produced by fibroblasts. It inhibits replication of virus in the host cell.

4.2.3.3. Cytotoxic lymphokines

Lymphotoxin (LT)

It is a cytotoxic protein released by activated Th-cells. It is also known as tumour necrosis factor-β(TNF-β). It is cytotoxic for foreign cells such as tumour cells, transplanted cells and microorganisms.

Tumour necrosis factor-α (TNF-α): It is produced by macrophages and monocytes. It causes lysis of tumour cells. It also plays a role in elimination of certain bacteria and parasites. It is also known as cachectin--causing cachexia-a wasting syndrome during chronic infections because of pronounced catabolic effects, e.g., breakdown of muscle protein.

4.2.3.4. Other Cytokines

Skin reactive factor (SRF): It is produced by specifically sensitised lymphocytes with Ags and mitogens. When injected into the skin of normal guinea pig, it produces an indurated and erythematous lesion within 3 hr. It facilitates the movement of monocytes from blood vessels into extravascular spaces and induces skin hypersensitivity.

Lymphocyte inhibitory factor: It has T-suppressor activity, suppresses Ab production.

Transforming growth factor-β(TGF-β): It is produced by T and B-cells. It is a growth factor that transforms fibroblasts and promotes wound healing. It inhibits T and B cell proliferation and haematopoiesis.

Leukaemia inhibitory factor (LIF): It is produced by T-cells. It helps in proliferation of stem cells and eosinophil chemotaxis.

Cytotoxic T-Cells: A population of killer (K) T-cells capable of killing or lysing target cells to which they bind are known as cytotoxic T-cells. They are formed in response to viral infection and graft from genetically dissimilar member. They are cytotoxic for host cells infected with virus, graft tissue and tumour cells. Mechanism of Action - The first

stage is the binding of effector cell (K-cell) to target cell, through specific receptors. It is a calcium independent stage. A change occurs in the target cell which leads to cytolysis. This phase is calcium dependent.

4.3. Summary

MHC class I and class II genes and their products provide a system for intracellular communication. The major function of MHC is antigen presentation and processing. MHC antigens are essential for recognition of antigen by T-cells. T-cells recognize antigen when it is presented in association with class I and class II histocompatibility antigens. T-helper cells with CD4 molecules recognize foreign antigen in association with class II antigen whereas cytotoxic T-cells with CD8 molecules are restricted by Class-I antigens. MHC class III products are the important components of complement system and are responsible for various cellular events. Cytokines are low molecular weight antigen-non specific proteins that mediate cellular interactions involving immuno, inflammatory and hematopoietic systems. The major functions of cytokines are to 1) Regulate specific immune responses 2) facilitate innate immuno responses, 3) activate inflammatory responses, 4) affect leukocyte movement, and 5) stimulate hematopoiesis.

4.4. Model Questions

Write an essay on major histocompatibility complex and its functions.

Give an account on cytokines and their role in different immune responses.

Write short notes on

a) MHC class I molecules
b) MHC class II molecules
c) Cytokines produced by macrophages
d) Lymphokines produced by Th 1 cells
e) Lymphokines produced by Th 2 cells

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LESSON NO. 5.

ANTIGEN AND ANTIBODY

5.0 Objectives : This lesson mainly emphasize on nature , structure and types of antigen and antibody.

5.1. Introduction

Antigen and antibody are the two important components of immune reactions which exhibit high specificity.

Definition Of Antigen : Antigen is defined as any substance, which, when introduced parenterally into the living animal body, evokes specific immune response either by producing specific antibody with which it reacts specifically and in an observable manner or by producing specifically sensitised T-cells or both. The antibodies or T-cells produced are reactive against the Ag. It is not necessarily microbes or their products but any substance foreign to the body may act as an antigen and stimulate specific immune response. Thus, any foreign protein like egg albumin, red, blood cells, vegetable proteins or snake venom may act as an antigen. There are two attributes of antigenicity. These are - **Immunogenicity:** It is the capacity to provoke specific immune response and

Immunological reactivity: It is the specific reaction of an antigen with antibody or specifically sensitised T-lymphocytes.

5.1.1. Types of Antigen (Ag)

Based upon the ability of antigens to carry out the two functions (attributes) mentioned above, antigens are classified into two types a) complete antigen and b) Hapten

a) Complete antigen (Immunogen): Antigen, which is able to induce antibody formation and able to react with it specifically and in an observable manner is known as complete antigen. The complete antigen is able to generate an immune response by itself and does not need a carrier molecule as in hapten. Thus, complete antigen can function as antigen as well as immunogen.

b) Hapten (Incomplete antigen): It is a substance, which is unable to induce antibody synthesis by itself but can react specifically with antibody. Thus, hapten can function as an antigen but not as an immunogen. Haptens are incomplete antigens, which become complete antigen when they covalently combine with carrier molecule or schlepper. After combination with carrier molecule, it becomes complete antigen and can induce immune response. Haptens are generally low molecular weight lipids and carbohydrates while carrier molecules are proteins such as serum albumin or globulin or synthetic polypeptides.

Examples of hapten are capsular polysaccharide of pneumococci, cardiolipin, polysaccharide 'C' of β-hemolytic streptococci, drugs like penicillin, etc.

5.1.3. Antigenic Determinants

Antigens are large molecules but only restricted portions on them are involved in the actual binding with antibody combining sites. Such areas, which determine the specific

immune response and react specifically with antibody, are known as antigenic determinants or epitopes. It is the smallest unit of antigenicity represented by a small area on the antigen molecule possessing a specific chemical structure and steric configuration. The number of antigenic determinants on an antigen molecule usually varies with its size and chemical complexity. An antigen possesses several epitopes, which differ in specificity and potency.

The properties of epitopes are

Size - 25-35 ^{0}A.

Molecular weight - 400-1000 Dalton.

The determinant groups

On protein Ag - penta or hexa peptide.

On polysacharide Ag - hexasaccharide.

A determinant is roughly five amino acids in size.

5.1.4. Properties of Antigen (Requirements of Immunogenicity/Factors Affecting Immunogenicity)

Antigens exhibit various properties like :

a) Foreignness: Recognition of self and non-self is an important function of immune system. Body does not give immune response to self antigens, so the first and most important requirement of immunogenicity is the foreignness of antigen. The antigen should be genetically foreign to the host. Substances that are foreign to the circulation are antigenic and able to mount an immune response. An individual normally does not respond to his own antigens. Thus, albumin isolated from the serum of a rabbit when injected back into the same rabbit will not induce antibody production but when injected into other animal, lead to formation of antibody.

In general, the antigenicity of a substance is related to the degree of foreignness. Antigens from other individuals of same species are less antigenic than antigens from

individuals of other species. Antigens from related species are less antigenic than antigens from distant species.

b) Size: In order to be antigenic, the substance must have certain minimum size. Extremely small molecules such as amino acids and monosaccharides are non-antigenic. Large molecules such as haemocyanin having molecular weight 6.75 million are highly antigenic. In general, substances having molecular weight less than 10,000, e.g., insulin (molecular weight--5000), are weakly antigenic or non-antigenic. The effective immunogens have molecular weight greater than 10,000. The substances having low molecular weight may be rendered antigenic by adsorbing them on large inert particles like bentonite or kaolin.

c) Chemical nature: Naturally occurring antigens are proteins and polysaccharides. Protein antigens are more effective than polysaccharide antigens. Lipids and nucleic acids are less antigenic. Their antigenicity can be enhanced by coupling them with proteins. Not all proteins are antigenic, for example, gelatin, histones and protamines are non-antigenic. The presence of an aromatic radical is essential for antigenicity. Non-antigenic substances can become antigenic after combination with aromatic radicals like tyrosine.

Chemically complex molecules are more antigenic, for example, synthetic polypeptides containing repeating units of single amino acid are weakly antigenic, however, polymers of two or three amino acids may be more antigenic. As a general rule, Antigenicity increases with structural complexity. The substances with diverse chemical and structural properties are the most powerful antigens. Presence of aromatic amino acid increases antigenicity. Simple polypeptides containing tyrosine are more antigenic than polypeptides without tyrosine.

d) Susceptibility to tissue enzymes: Substances that are metabolized and susceptible to the action of tissue enzyme are good antigens. Antigen administered into the body is processed by phagocytic cell and intracellular enzymes into appropriate fragments

containing epitopes, which are responsible for immune response. Substances such as polystyrene latex and synthetic polypeptides composed of d-amino acids, which are not susceptible to tissue enzymes are not metabolized in the body, are non-antigenic; whereas polypeptides made up of l-amino acids are antigenic. The processing of antigen is necessary to expose the hidden determinants of antigen.

e) Genetic constitution of the host: The immune response is genetically controlled phenomenon, so the genetic makeup of the host plays an important role in the development of immune response. The ability of the host to respond to a particular antigen varies with genetic make-up of the host. The immunogenicity of antigen varies from species to species and even from individual to individual. It has been observed that the pure polysaccharides are antigenic when injected into mice and human beings, but not when injected into guinea pigs. This is because of the difference in genetic makeup. When all physical and chemical requirements for immunogenicity are fulfilled, the capacity of an individual to respond to various immunogens is genetically determined.

5.1.5. Antigenic Specificity

The reaction between antigen and antibody is highly specific. An Ag reacts with its corresponding antibody only and vice-versa. Antigenic specificity depends upon the specific active sites on the antigen molecules, i.e., antigenic determinants. The nature of these determinants is not exactly known. The basis of antigenic specificity was studied by Karl Landsteiner. In his experiment, he found that the antigenic specificity is determined by single chemical groupings and even by a single acid or basic groups that are important in regulating the specificity of an antigenic determinant. Spatial configuration of the determinant group, as evidenced by differences in antigenic specificity of *dextro, levo,* and *meso* isomers of substances such as tartaric acid. Spatial distribution of the radicals into the antigenic molecule, as evidenced by the differences in specificity in a compound with the group attached at the *ortho, meta* and *para* positions. For example, *ortho, meta* and *para* amino benzoic acids are antigenically different. Terminal groups in an antigen molecule are also important determinants.

Antigenic specificity is not absolute and cross reactions may occur between similar or related antigens due to stereo-chemical similarities and sharing of antigenic determinants by different antigens.

The specificity of natural tissue antigens of animals may be considered under different categories. These are species specificity; isospecificity; organ specificity; and heterogenetic specificity.

5.1.5.1. Species specificity: Tissues of all members in a species contain species specific antigen. For example, human blood proteins are antigenically different from animal blood proteins. The species specific antigens show some degree of cross reaction with antigens from related species. The immunological relationship is similar to phylogenetic relationships. These immunological relationships between species specific antigens are useful in tracing of evolutionary relationship ; in the identification of the species of blood and of seminal stains (forensic application); to study incidence of cross reactions between antigens from different species, which result in hypersensitivity.

5.1.5.2. Isospecificity: Isoantigens, also known as alloantigens, are antigens found in some, but not all members of a species. Based on the presence of different isoantigens, members of species may be grouped into different groups. For example, Human erythrocyte antigens - based on which individuals are classified into different blood groups as A,B, AB and O. These antigens are genetically determined and are important in blood transfusion ; isoimmunization ; in providing valuable evidence in disputed paternity and also useful in anthropology. Other examples of isoantigens are histocompatibility antigens associated with plasma membrane of tissue cells. These are species specific antigens but show a difference individual members of the same species, so that transfer of tissue cells between members induce an immune reaction.

5.1.5.3. Autospecificity: The autologous or self antigens are generally not antigenic, but there are a few self antigens which are not exposed to immune system because of their sequestered or hidden nature. For example, eye lens protein, thyroglobulin, etc., are anatomically confined to sites that prevent their contact with immune system, so they are not recognized as self antigens and also the antigens that are absent during the embryonic

life and appear in later life, for example, sperms, are also not recognized as self antigens. When these antigens are released into circulation following injury to eye lens or damage to thyroid or testis and come in contact with immunocompetent cells, the result is formation of autoantibody producing autoimmune disease. The antigenic specificity of self antigen may be altered as a result of infection, irradiation or drug therapy and hence may become immunogenic.

5.1.5.4. Organ specificity: Organ specific antigens are antigens confined to a particular organ or tissue. Organs such as brain, kidney, thyroid, eye lens of one species share specificity with another species. Such antigens present in an organ or tissue of different species are called organ specific antigens. For example, brain-tissue antigen of man, shares antigenicity with brain tissue antigen of sheep. This sharing of antigens results in neuroparalytic complications in man following the antirabies vaccine containing partially denatured sheep brain tissue. The sheep brain-tissue antigen induces antibody formation that cross reacts with human brain-tissue and produces damage.

e) Heterogenetic or heterophile specificity: The similar or closely related antigens present in different biological species, classes and kingdoms are known as heterogenitic or heterophile antigens. Antibodies to these closely related antigens produced by one species. The best known example is the Forssman antigen (Forssman 1911). It is a lipoprotein polysaccharide complex, which is widely distributed in man, animals, birds, plants and bacteria. It is present in red blood cells and other tissues of the animals such as horse, cat, mouse, sheep, chicken and kidney cells of guinea pig. It is also present in certain bacteria such as pneumococci, some *Salmonella* serotypes, *Shigella dysenteriae, Pasteurella, Cl. welhilli and Br. catarrhalis.* Other examples of heterophile antigens (cross reacting antigens) are – i) a heterophile antigen of Rickettsiae causing typhus fever shared by certain strains of *Proteus* (OX 19, OX 2 and OX K). This forms the basis of Weil-Felix reaction in which *Proteus* strains are agglutinated by heterophile antibody in serum of a patient suffering from typhus fever. ii) Epstein-Barr virus causing infections mononucleosis shares antigenicity with sheep and ox RBCs (Paul-Bunnel Test). iii) In primary atypical pneumonia human 'O' RBCs are agglutinated by patients serum (cold agglutination test). iv) Human 'B' RBCs share antigen with *E. coli.* v) Blood Group 'A'

substances share antigen with capsular polysaccharide of pneumococcus type 14 and streptococcal extracts.

5.2.Definition of Antibody

The immunoglobulins/antibodies are a group of glycoproteins present in the serum and tissue fluids of all mammals. With the possible exception of natural antibody, they are formed in response to foreign substance (antigen) administered into the body with which they react specifically and in an observable manner.

5.2.1. Nature of antibody : Antibodies are globulins in nature and are known as immunoglobulins as they are involved in immune reactions. They contain sugar residues and hence are glycoproteins. Serum globulins can be separated into pseudoglobulins (water soluble) and euglobulins (water insoluble). Most antibodies are euglobulins. Immunoglobulins constitute 20-25% of the total serum proteins. When electrophoretically separated, most of the serum antibodies migrate in gamma region hence they are also termed as gammaglobulins. Many immunoglobulins also migrate in beta region and even in alpha region. Sedimentation studies showed that most antibodies belong to 7S (Svedberg unit) class having molecular weight 1,50,000-1,80,000 and some belong to 19S having molecular weight 9,00,000 and are designed as M or macroglobulins. Of the different terms were used earlier, the generic term immunoglobulin is internationally accepted for 'proteins' of animal origin endowed with known antibody activity and for certain other related proteins. The term immunoglobulin denotes chemical structure of protein while antibody refers to biological activity and function of proteins. Accordingly, immunoglobulins include abnormal plasma proteins found in myeloma, macroglobulinemia, cryoglobulinemia and the naturally occurring subunits of immunoglobulins in addition to antibody globulins. Thus, all antibodies are immunoglobulins but all immunoglobulins may not be antibodies. Immunoglobulins are mainly synthesized by plasma cells and to some extent by lymphocytes.

5.2.2. Structure of Immunoglobulin (Fig. 5.1): The detailed structure of immunoglobulin was studied by Porter *et al*.(1962) by cleaving immunoglobulin molecule. They used rabbit IgG antibody to egg albumin for their study. The IgG can be

digested by papain in the presence of cysteine into three fragments. Out of three, two are identical having molecular weight 45,000 (sedimentation co-efficient 3.5S) and are able to combine with antigen but unable to precipitate the reaction. Therefore, these are univalent and are called Fab (fragment antigen binding). The third fragment has no power to combine with antigen (molecular weight 55,000) and is termed as Fc (fragment crystallizable). Fab contains a light chain and a part of heavy chain, and Fc contains part of heavy chain only. When treated with another proteolytic enzyme-pepsin, a 5S fragment composed of two Fab fragments held together in position is obtained. It is bivalent and precipitates with antigen. This fragment is known as $F(ab)_2$. The Fc portion is completely degraded by pepsin into smaller fragments. Immunoglobulins can also be broken down into peptide chains by reduction with sulphydril reagent such as 2-mercaptoethanol. Based on these findings, Porter *et al*. put forward a basic four-chain model for immunoglobulin containing two distinct types of polypeptide chains-two heavy chains and tow light chains linked together by disulfide bond (-s--s-) and non-covalent linkages.

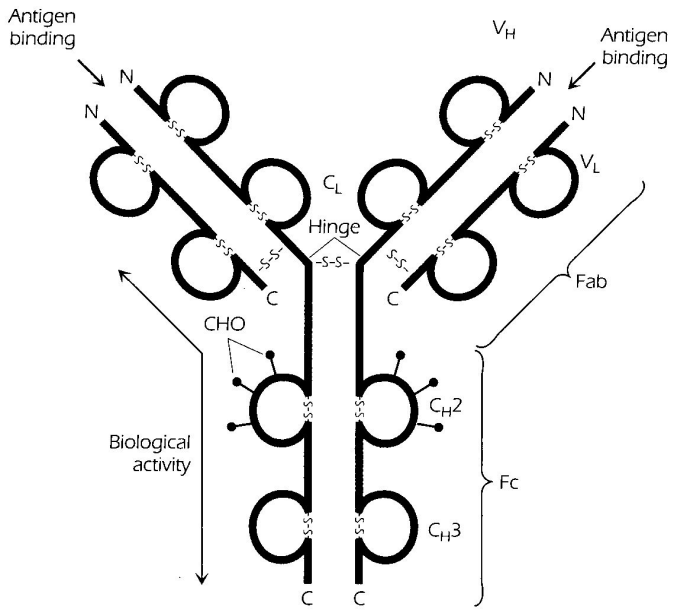

Fig.5.1 Structure of immunoglobulin showing domines formed by interchain disulphide bonds

a) Light chain (L-chain): These are smaller chains with molecular weight of 20,000--25,000 D, made up of 210 to 230 amino acids. This chain is attached to H-chain by disulfide bonds. L-chains are similar in all classes of immunoglobulins. They occur in two groups called *kappa* (κ) and *lamba* (λ). Immunoglobulin molecule may have either κ or λ, but never both together. κ and λ occur in a ratio of 2:1 in human sera. About 60% of molecules possess κ-type and about 30% of molecules possess λ-type.

b) Heavy chain (H-chain): These are large chains with molecular weight of 50,000 D. containing 420 to 460 amino acids. The two H-chains are joined together by one to five S--S bonds. H-chains are structurally and antigenically different for each class of immunoglobulin and are designated by Greek letters as follows.

Ig Class	Type of H-chain
Ig G	γ (Gamma)
IgM	μ (Mu)
IgA	α (Alpha)
IgD	δ (Delta)
IgE	ε (Epsilon)

c) Variable and constant region: Each polypeptide chain of immunoglobulin molecule contains an amino terminal end, which means there is a free amino group on the terminal amino acid. This is called variable region (V) which is different for each class and subclass. The first 110 amino acids from the amino terminal end are responsible for antibody reactivity. Each polypeptide chain contains carboxy terminal end, which means there is a free carboxyl group on the terminal amino acid. This is called constant (C) region whose composition is constant in all immunoglobulin molecules from different animal species except for some minor genetic differences.

d) Functions of Fab and Fc: The antigen binding site (Fab) of the antibody molecule resides at amino terminal end. It is composed of both L and H chain. The portion of H-chain present in Fab fragment is known as Fc piece. The Fc fragment resides at carboxy

terminal end. It does not possess antigen binding site but determines the biological properties of the immunoglobulin molecule such as - Complement fixation, Placental transfer, Skin fixation, Catabolic rate, Secretion into body fluid, Binding to phagocytes, and Binding to mast cells. The antibody specificity of immunoglobulin molecule depends on the variability of the amino acid sequences at the variable region of the H and L chain, which form the antigen binding site. The antibody specificity is explained on the basis that the combining site of antibody molecule possesses a specific amino acid composition that is complementary fit for specific reactive area of the antigen molecule. Recently, hypervariable regions (paratopes) in variable portions of H and L chains have been identified. In L-chains these regions are L1 (residues 23-36), L2 (52-58), and L3 (91-99). In H-chains these are H1 (from 31 to 36), H2 (from 49 to 66), and H3 (from 99 to 104). These hypervariable regions (hot spots) are involved in the formation of antigen binding site.

e) Hinge region: When antibody molecule is visualized under electron microscope, it appears as a Y-shaped structure whose arms can swing out to an angle of 180^{o} through the papain and pepsin sensitive region called hinge region. Hinge region consists of a large number of proline residues.

f) Immunoglobulin domains: The polypeptide chains do not exist as a linear sequence of amino acid molecules but are folded by disulfide bonds into globular regions known as domains. The domains of H-chain are designated as CH-1, CH-2, CH-3, CH-4 (domains in constant region), and VH (domain in variable region). The domains of L-chains are designated of CL (Constant region) and VL (Variable region). Each domain serves a different function.

1. The VL and VH domains are responsible for the formation of specific antigen binding site.
2. The CH-2 domain in IgG binds to Clq in classical complement pathway.
3. CH-3 domain mediates adherence to the surface of monocytes.

5.2.3. Immunoglobulin Classes (Fig. 5.2.): Human sera contain five different immunoglobulins---IgG, IgA, IgM, IgD, and IgE---in the decreasing order of concentration.

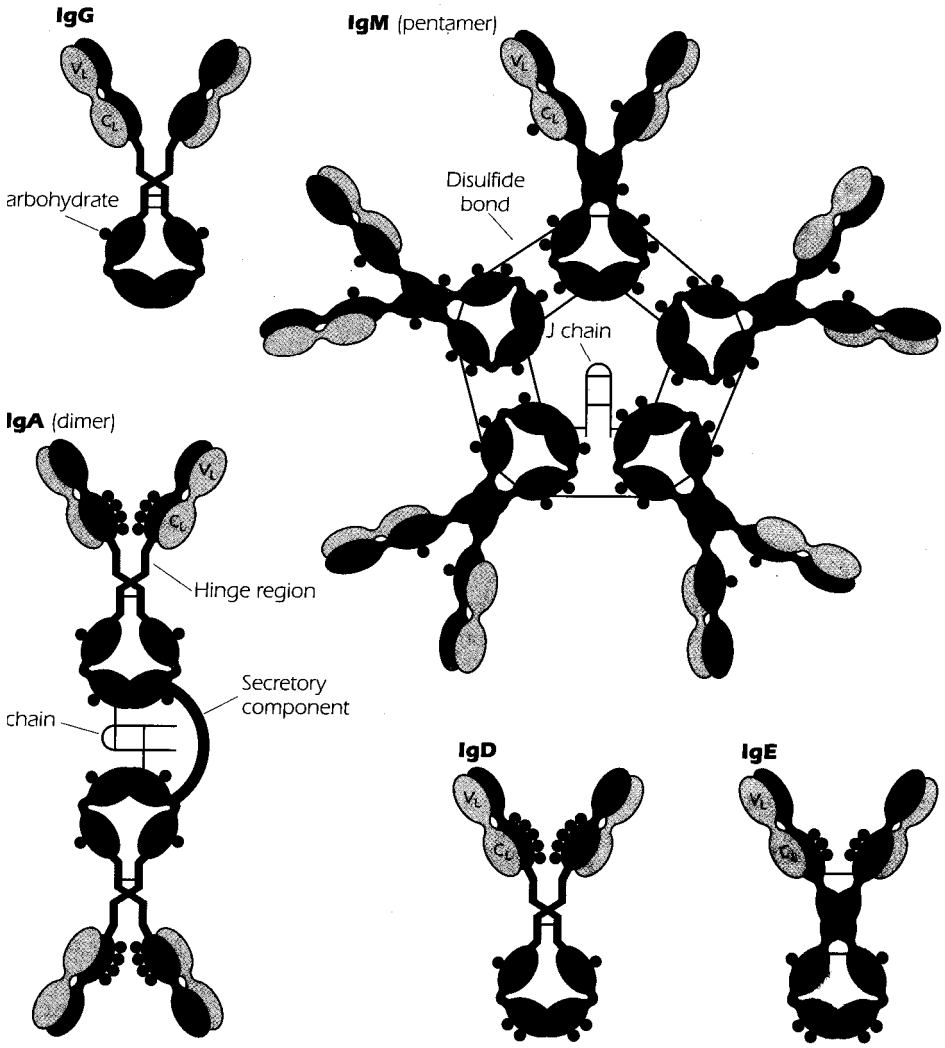

Fig 5.2. Structure of five different immunoglobulin molecules

i) Immunoglobulin-G (IgG)

General properties

1. It is the major immunoglobulin in normal serum accounting for 70---80% of the total.
2. It is a monomer consisting 2H and 2L chains.
3. Molecular weight ---1,50,000.
4. Sedimentation coefficient ---7S.
5. Half-life ---23 days.
6. Contains less carbohydrates ---3%.
7. Valency---2.
8. Normal serum concentration---8 -16mg/ml.
9. IgG subclasses---four classes - IgG1(65%), IgG2(23%), IgG3(8%), and IgG4(4%). Each subclass possesses different type of H-chain designated as λ-1, λ-2, λ-3 and λ-4.
10. It is the major immunoglobulin synthesized during secondary response.
11. It is equally distributed between the intravascular and extra vascular compartments.
12. It possesses greater antigen binding affinity than IgM.
13. Catabolic rate---when its level in serum is raised, as in chronic malaria, kala-azar or myeloma, the IgG is rapidly catabolized, however, in hypogammaglobulinemia it is slowly catabolized.

Biological activities

1. Because of its ability to cross the placenta, it provides a major line of defense (naturally acquired passive immunity) against infection in newborn for the first few weeks.
2. In extravascular body spaces, it carries the major burden of toxin neutralization.
3. It binds to microorganisms and enhances their phagocytosis.
4. It is able to activate complement and thus helps to attract polymorphonuclear leucocytes (phagocytic cells) by chemotactic mechanism and stimulates ingestion and killing of microorganisms.

5. IgG with the help of Fab reacts with target cell and mediates extracellular killing by K-cells bearing the specific receptor, which react with Fc portion of IgG on target cell and kill it .
6. It participates in most of the immunological reactions such as complement fixation, precipitation and neutralization of toxins and viruses.
7. It participates in allergic reactions, e.g., Arthus reactions and also in autoimmune diseases.
8. When administered passively, it suppresses the homologous antibody synthesis by a feedback mechanism. This property is utilized in the isoimmunization of women during delivery by the administration of anti –Rh (D) IgG.

ii) Immunoglobulin A (IgA)

General properties

1. It is the second most abundant immunoglobulin constituting 10--13% of the total.
2. It occurs in two forms--in human sera more than 80% of IgA occurs as monomer containing 2H and 2L chains but in most mammals it is polymeric.
3. Molecular weight--1,60,000.
4. Sedimentation coefficient---7S.
5. Half-life---6-8 days.
6. Carbohydrate content---11%.
7. Valency---2 or multiples of 2.
8. Normal serum concentration - 0.6 - 4.2 mg/ml.
9. IgA subclasses---2 classes --- IgA1 and IgA2. based on type of heavy chain (α-1 and α-2).

Secretory IgA

1. It is the predominant immunoglobulin present in the seromucous secretions such as saliva, tears, nasal fluids, sweat, colostrum and secretions of the lungs, genitourinary and gastrointestinal tract.
2. It is a dimer containing 4H and 4L chains.
3. Molecular weight---3,85,000.
4. Sedimentation coefficient---11S.
5. Possesses a cysteine rich polypeptide chain called J-chain (joining chain) of molecular weight 15,000 that joins two monomeric units of IgA. The J-chain is synthesized by same cells synthesizing dimeric S-IgA.
6. Possesses an additional structural unit - a glycine rich polypeptide called the T (transport) or S (secretory piece or secretory component) having molecular weight 60,000, synthesized by epithelial cells and is attached to the IgA molecule during transport across the cells. It appears to protect IgA from digestion by proteolytic enzymes (Fig. 5.3).

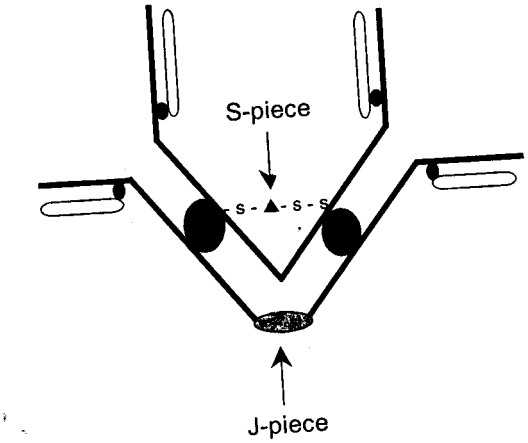

Fig. 5.3 Secretory Ig A molecule

Biological activities

1. IgA present in secretions is synthesized locally by plasma cells situated near the mucosal or glandular epithelium and selectively concentrated in secretions and on

mucous surfaces forming an antibody paste. This IgA plays an important role in local immunity against respiratory, intestinal and urogenital pathogens by inhibiting the adherence of microorganisms to the surface of mucosal cells by coating them and thereby preventing their entry into the body tissue.

2. Can activate complement by alternate pathway and helps to kill certain coliforms with the help of lysozyme.
3. Promote phagocytosis and intracellular killing of microorganisms.
4. IgA has also been reported to mediate antibody dependent T-cell mediated cytotoxicity.
5. The potential functions of S-IgA are inhibition of bacterial adherence, virus and toxin neutralization and prevention of antigen uptake by epithelial cells.

iii) Immunoglobulin M (IgM)

General properties

1. It is the first antibody formed in every antibody response.
2. Constitutes 5-8% of serum immunoglobulins.
3. It is a pentamer containing 10H and 10L chains--five subunits of monomer (Fig. 5.4).
4. Possesses J-chain (molecular weight 15,000) responsible for joining of subunits.
5. It bears an extra C_H domain.
6. Molecular weight 9,70,000, hence known as 'the millionaire molecule'.
7. Sedimentation coefficient--19S.
8. Half-life---5 days.
9. Carbohydrate content---10%.
10. Valency--theoretically---10 (observed with small haptens). The effective valency is five due to steric hindrance.
11. Normal serum concentration--- 0.5 - 2mg/ml.
12. IgM subclasses---two subclasses--IgM1 and IgM2, based on H-chains(μ-1 and μ-2).
13. Most of the IgM (80%) is intravascular in distribution.
14. Susceptible to mercaptoethanol---serum treatment with mercaptoethanol selectively destroys IgM.

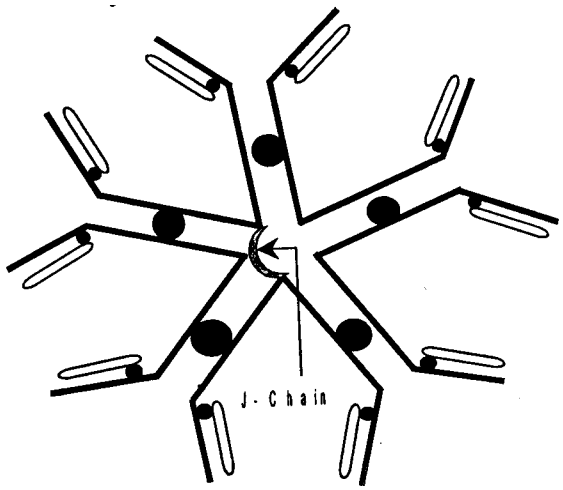

Fig. 5.4. Ig M Molecule

Biological activities

1. Because of high valency, it is extremely efficient in agglutination and cytolytic activity.
2. As IgM is largely confined to blood stream, it offers protection against bacteraemia.
3. As it is not transported across the placenta, its detection in foetus or new born indicated intrauterine infection, which is useful in the diagnosis of congenital syphilis, rubella, toxoplasmosis and HIV infection.
4. It fixes complement by classical pathway.
5. It is the most efficient antibody in agglutination, complement fixation and cytolytic reaction.
6. It is more effective than IgG in immune haemolysis, opsonization and bactericidal action.
7. It also neutralizes viruses and toxins but less efficient than IgG.
8. As it is a short lived immunoglobulin that disappears rapidly, its demonstration in serum indicates recent infection.
9. Monomeric IgM appears on the surface of unstimulated B-lymphocytes and acts as recognition receptor for antigens.

iv) Immunoglobulin D (IgD)

General properties

1. Accounts for less than 1% of the total immunoglobulin.
2. Structurally, it resembles IgG--it is a monomer containing 2H and 2L chains.
3. Molecular weight---1,84,000.
4. Sedimentation coefficient--7S.
5. Half-life---2 - 8 days.
6. Carbohydrate content---13%.
7. Valency---2.
8. Normal serum concentration -- 0-0.04mg/ml.
9. Subclasses — two -- IgD1 and IgD2.
10. Susceptible to proteolytic degradation.

Biological activities

IgD together with IgM may function as antigen receptor on the surface of B-lymphocytes for recognition of antigens and for the control of lymphocyte activation and proliferation to produce antibodies or suppression.

v) Immunoglobulin E (IgE)

General properties

1. Occurs in very low concentration.
2. Structurally, it resembles IgG---it is a monomer containing 2H and 2L chains.
3. Molecular weight---1,88,000.
4. Sedimentation coefficient--8S.
5. Half-life---2 -3 days.
6. Carbohydrate content---12%.
7. Valency---2.
8. Normal serum concentration - occurs in very low concentration - 0.00003mg/ml but the level is greatly elevated in atopic conditions such as asthma, hay fever and eczema.

9. Heat labile---inactivated at 56^{o}C in one hour.
10. Susceptible to mercaptoethanol.
11. Mostly extravascular in distribution.

Biological activities

1. It has affinity towards the surface tissue cells, particularly mast cells and basophils and is responsible for degranulation of these cells, thereby releasing vasoactive amines and other mediators. Thus, it is responsible for Type-1 hypersensitivity reactions (hay fever, infantile eczema, asthma and atopic dermatitis).

2. The IgE on the mast cell surface triggers the release of vasoactive agents and factors chemotactic for granulocytes. The vasoactive agent causes the transudation of IgG and complement while chemotactic factors attract effector cells (neutrophils and eosinophils) needed to dispose the infectious agent coated with IgG and complement. Thus, it plays an important role in immunity against helminthic parasites.

5.2.4. Summary

Antigen is defined as any substance, which, when introduced parenterally into the living animal body, evokes specific immune response either by producing specific antibody with which it reacts specifically and in an observable manner or by producing specifically sensitised T-cells or both. Immunogenicity and Immunological reactivity are the two attributes of Antigenicity Based upon the ability of antigens to carry out the two functions, antigens are classified into two types a) complete antigen and b) Hapten. The effective immunogens have molecular weight greater than 10,000.

Immunoglobulins are glycoproteins. The basic unit of the molecule is a four chain monomer. These four polypeptide chains consists of two identical heavy chains and two identical light chains, designated as H and L chains respectively. Each polypeptide chain consists an amino terminal and a corboxy terminal. The amino terminal of the molecule is part of the variable or 'V' regions (V_L and V_H) and corboxy terminal portion the constant or C regions (C_H and C_L). The C_H region is further divided into three areas CH_1, CH_2,and CH_3 . There are mainly five classes of immunoglobulins based on the type of the heavy

chains. They are IgG, IgD, Ig E, IgM and IgA named after the types of heavy chains gamma, delta, epsilon, mu, and alpha respectively. IgG protects the body fluids. IgM protects the blood stream. Ig E mediates reagenic hypersensitivity. IgA protects the body surface. IgD together with IgM may function as antigen receptor on the surface of B-lymphocytes for recognition of antigens.

5.3. Model Questions

1) Write an account on nature, structure and functions of antigen.
2) Discuss the structure and types of immunoglobulins and add a note on their functions.

Write short notes on the following

a) Types of antigens
b) Chemical nature of antigen
c) Properties of antigens
d) Types of immunoglobulins
e) Sub classes of immunoglobulins.
f) Biological functions of immunoglobulins

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LESSON NO. 6

IMMUNE REACTIONS

6.0. Objectives : In this lesson different important types of antibody and antigen reactions like precipitation, agglutination, neutralization and complement fixation, were described in detail.

6.1. Introduction

When an antigen solution is mixed in a correct proportion with a potent antiserum, an antigen antibody complex is formed. This complex formation in an observable manner due to combination of specific antigen with specific antibody is called antigen-antibody reaction.

$$Ag + Ab \rightarrow Ag\text{-}Ab\ Complex.$$

Importance of Ag-Ab Reactions : In the body, they play an important role in Ab-mediated immunity against infectious diseases and tissue injury in hypersensitivity and autoimmune disease.

In the laboratory, Ag-Ab reactions are useful - in the diagnosis of infections ; in epidemiological surveys; in the identification of infectious agents; and in the identification of non-infectious agents such as enzymes. In general, these reactions can be used for the detection and quantitation of either antigens or antibodies.

By definition the Ag-Ab reactions in vitro are known as serological reactions.

Characteristics of Ag-Ab Reactions: The reaction between Ag and Ab is highly specific. This specificity, however, is not absolute and cross-reactions between related or similar Ags may occur. The antigenic determinant makes contact with an area on the hypervariable region called paratope of the Ab. The molecules are held together in lock and key arrangement by spatial complementarity and not by covalent bonding. Entire molecules interact and not fragments. Though only antigenic determinant is involved in actual binding, the whole molecules or particles are agglutinated. The combination occurs at the surface, so only surface antigens participate during combination. The combination is firm but reversible. The firmness is affected by affinity and avidity of the reaction. Affinity is the intensity of attraction between antigen and antibody and avidity is the binding strength of the individual Ab with its specific antigenic determinant. Ags and Abs can combine in varying proportions due to their valences. Abs are generally bivalent and Ags may be multivalent.

6.1.1. Stages of reactions

The reactions between Ag and Ab occur in three stages.

i) Primary stage: It is the initial interaction between Ag and Ab without any visible effects. The reaction is rapid, occurs even at low temperature and obeys the general laws of physical chemistry and thermodynamics. The reaction is reversible and weak because of weak intra-molecular forces such as Van der Waal's forces, ionic bonds and hydrogen bonds. (No covalent bonding), e.g., Coomb's test, fluorescent Ab tests.

ii) Secondary stage: The primary stage in most instances, but not all, results into secondary stage leading to demonstrable events such as precipitation, agglutination, lysis of cells, killing of live antigens, complement fixation, neutralization of toxins and other Ags, inhibition of motility, enhancement of phagocytosis, etc.

iii) Tertiary stage: Some Ag-Ab reactions in vivo initiate chain reactions leading to neutralization or destruction of injurious Ags or tissue damage. These are the tertiary reactions responsible for humoral immunity against infectious diseases, hypersensitivity reactions and other immunological diseases.

6.1.2. Measurement of Ag and Ab: Ags and Abs participating in the primary, secondary, or tertiary reactions can be measured by different methods. Measurement may be in terms of mass, e.g., mg or more commonly as units or titer. The titer is defined as the highest dilution of the serum, which gives an observable reaction (positive reaction) with the Ag in the particular test. Ags can also be titrated against sera (Ab).

Two important parameters of serological tests are sensitivity and specificity.

i) Sensitivity: It is the ability of the test to detect even very minute quantities of Ag or Ab. When a test is highly sensitive, false negative results will be absent or minimal.

ii) Specificity: It is the ability of the test to detect reactions between homologous Ags and Abs only. In a highly specific test, false positive reactions will be absent or minimal.

6.2. Types of antigen and antibody reactions

6.2.1. Precipitation and Flocculation

Definition**:** When a soluble Ag (precipitinogen) combines with its antibody (precipitin) in the presence of electrolytes (NaCI) at a suitable temperature ($37^{o}C$) and pH(7.4), the Ag-Ab complex is formed as an insoluble precipitate. This reaction is called **precipitation**.

Precipitinogen + Precipitin $\rightarrow$ Insoluble precipitate

(Soluble Ag) (Ab) (Ag-Ab complex).

Flocculation: It is a precipitation reaction in which the precipitate of Ag-Ab complex remains suspended as floccules instead of sedimentation.

Principle :

Zone phenomenon

The amount of precipitate formed is greatly influenced by the relative proportions of Ags and Abs. When increasing quantities of Ag are added to the fixed amount of antiserum, precipitation occurs most rapidly and abundantly in one of the middle dilutions in which the Ag and Ab are present in optimum proportions. In the first few dilution in which the Ab is in excess and in the last few dilutions in which the Ag is in excess, the precipitation will be minimal or absent. If the amounts of precipitate at different dilutions are plotted, the resulting curve will have three different phases.

An ascending zone: A zone of Ab excess or prozone in which some uncombined Ab is present.

A peak: A zone of equivalence in which Ag and Ab are completely precipitated.

A descending zone: A zone of Ag excess or postzone in which all Abs have combined with Ag but some uncombined Ag is present.

This is known as zone phenomenon. Zoning occurs in agglutination and some other reactions also. The prozone is very important, as sera rich in Ab may give a false negative precipitation or agglutination reactions.

Mechanism of precipitation

The first step is linking together of different antigens by Ab molecules that specifically attach to the antigenic determinants on the surface of Ag. Marrack (1934) proposed that each Ab which is divalent forms a bridge between two Ag molecules. Ag being multivalent can combine with a number of Ab molecules. This combination results in the formation of multimolecular lattice, which makes the reaction visible. It is known as *lattice hypothesis.* This theory requires that - The Ab should be divalent at least and Ag and Ab in optimum proportion.

Precipitation occurs when Ag and Ab react in equivalent proportion (zone of equivalence). The lattice formation does not occur in the zones of either Ag or Ab excess.

Applications: Precipitation reaction is very sensitive for detection of Ag and can detect as little as one μg of protein antigen but comparatively less sensitive for the detection of Abs. The test may be carried out either as a qualitative test or quantitative test. The qualitative precipitation test is widely used for detection of antigens and is particularly valuable in - Identification of bacteria, Identification of bacterial components in infective tissues, Detection of unknown antibody, Medicolegal identification of human blood or seminal fluid and Standardization of toxins and antitoxins.

6.2.1.1. Techniques of precipitation and flocculation reaction

Precipitation reaction can take place in a liquid medium or in semisolid medium (gels) such as agar gels, agarose or polyacrylamide.

The following are the types of precipitation and flocculation tests.

6.2.1.1.1. Ring test: In this test, antiserum is placed at the bottom of a narrow bottom tube and an antigen solution (an extract of the organism) is layered over it. A white ring of precipitate forms at the junction of two fluids. This is the simplest type of precipitation employed for the detection of Ag, for example, Ascoli's thermo precipitation test for anthrax ; C-reactive protein test; The grouping of streptococci by Lancefield technique.

6.2.1.1.2. Slide flocculation test: When a drop of antigen solution and patient's serum are placed on a slide and mixed well by shaking, the result is formation of floccules in positive cases. For example, VDRL test for diagnosis of syphilis.

6**.2.1.1.3**. **Tube flocculation test:** Here, instead of slide, the test is performed in tube. An antigen and serum are placed in a tube and mixed by shaking. This results in formation of floccules. For example, Kahn test for diagnosis of syphilis. It is also used for the standardization of toxins and toxoids.

6.2.1.1.4. Immunodiffusion test: It is a precipitation reaction carried out in a gel. Precipitation reaction carried out in gel is more advantageous than precipitation in a liquid medium. The advantages are - The gel has got certain porosity through which antigens and antibodies migrate to form precipitation band where they meet in optimum proportion. The band is stable and can be preserved by staining, if necessary. Each antigen-antibody reaction results in the formation of a distinct band of precipitation, therefore the different antigens in the reacting mixture can be observed and detected. Immunodiffusion also indicates identity, cross-reaction and nonidentity between different antigens.

Requirements: Gelling agent--agar (1%), agarose, polyacrylamide. Buffers such as barbitone buffer (pH--8.6). Preservatives--sodium azide, merthiolate, zephiran or copper sulphate to prevent bacterial and fungal growth.

i)Types of immunodiffusion

a) Single diffusion in one dimension (Oudin procedure): In this test, one of the reactants is stationary while other moves by diffusion. The antibody is incorporated in agar gel at 5.6 ^{o}C in a test tube and allowed to solidify. The antigen solution is layered over it. The antigen diffuses downward, reacts with antibody and forms precipitation band. The number of bands indicate the number of Ags in a mixture. The test is used for detection of number of Ags present in a mixture.

b) Double diffusion in one dimension (Oakley-Fulthorpe procedure): In this test, both Ag and Ab move towards each other by diffusion. The Ab is incorporated in agar gel and placed at the bottom of tube. Above this, a column of plain agar is placed. The Ag solution is layered on top of the column of plain agar. Ag and Ab move towards each other through the column of plain agar and form a band of precipitate. This test was used to determine the number of antigens in a mixture.

c) Single diffusion in two dimensions (Radial immunodiffusion): In this test, Ab is stationary while Ag move in two dimensions. The Ab is incorporated in agar gel at 56^{o}C and poured on a glass plate or petridish. The gel is allowed to cool and set. Wells are punched. Few wells are charged with known concentration of Ags and remaining with unknown. The Ag moves radially from the well and react with Ab in gel forming ring shaped band of precipitation. The diameter of the ring (halos) is directly proportional to the Ag concentration and gives an estimate of the concentration of the antigen (Fig. 6.1). This test is used - For the quantitation of soluble Ags in body fluids; for the quantitation of immunoglobulins, plasma proteins and complement components; for the quantitation of Abs and proteins in other body fluids such as CSF (cerebrospinal fluid), urine, milk, colostrum, etc.

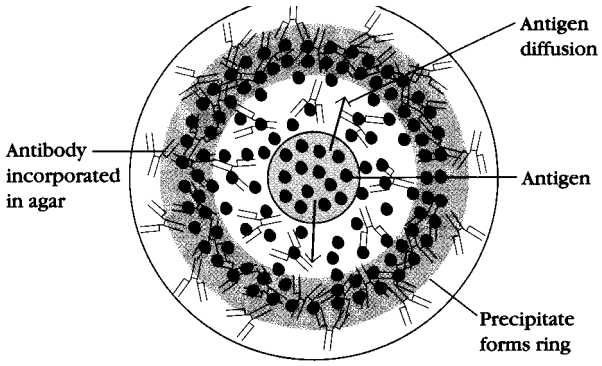

Fig. 6.1. Radial immunodiffusion

d) Double diffusion in two dimensions (Ouchterlony procedure): It is also known as agar gel diffusion (Fig 6.2.). In this test, Agar gel is poured on a plate or a slide and allowed to solidify. Wells are punched using template (3-4mm in diameter). The antiserum is placed in the central well and different Ags are placed in the surrounding wells. The slide is then placed in a moist chamber to prevent drying and incubated for 24 hr. Observed for precipitate. If two adjacent Ags are identical, the lines of precipitate formed will fuse. If they are not identical, the lines will cross each other. Cross reaction or partial identity is indicated by spur formation. This test is used -To detect an Ag; to compare two Ag, Ab systems; in the diagnosis of bacterial diseases, for example, Elek test for toxigenicity testing in diphtheria ; In the diagnosis of viral diseases such as small pox, HBV, influenza, polio, etc.; in the diagnosis of fungal diseases such as candidiasis, cryptococcosis, histoplasmosis, etc.; in the diagnosis of parasitic diseases such as malaria, amoebiasis, ascariasis, hydatid disease, etc.

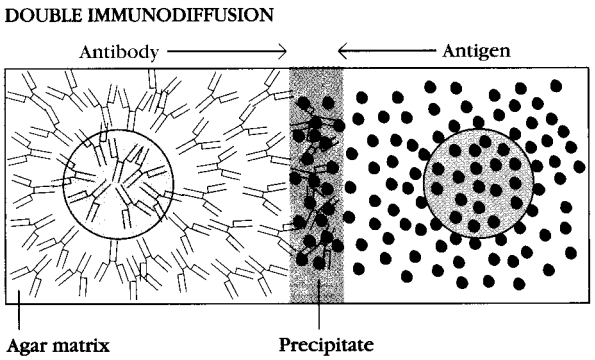

Fig. 6.2 Double immunodiffusion

e) Immunoelectrophoresis (IEP): It is a combination of electrophoresis and agar gel diffusion. In the first step, agar is poured on plate or a slide and allowed to solidify. Well is punched and charged with sample. Electric current is applied fro 30-60 min and individual components (different Ags) are separated according to their charge and size. (Fig. 6.3) In the second step, a trough is cut parallel to and slightly away from the path of electrophoretic separation. Trough is filled with antiserum and placed in a moist chamber to allow diffusion for 18-24 hr. The Ag and Ab diffuse towards each other and form arcs of precipitate where they meet. Each are indicates different type of protein. This method is used for - Analysis of normal human serum; to detect abnormalities in human sera in diseases such as myeloma, heavy chain disease, cancer, etc.; and to detect abnormal proteins in various body fluids.

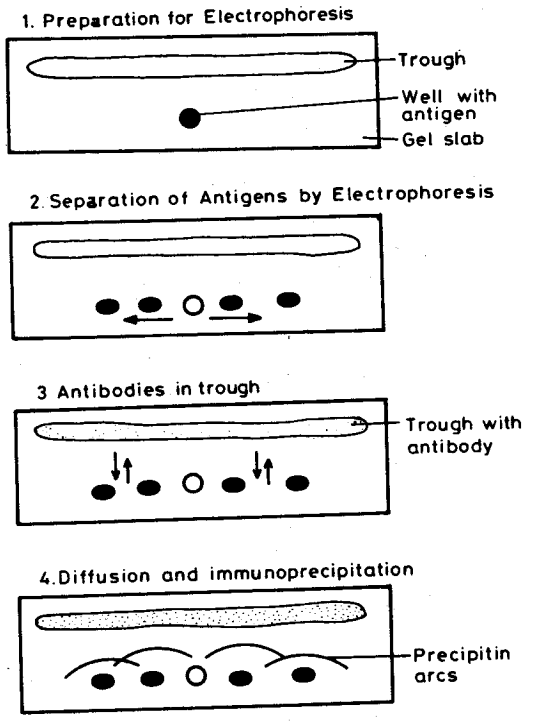

Fig.6.3 Immunoelectrophoresis

f) Counter current immunoelectrophoresis (CIEP) [Counter immunoelectrophoresis (CIE)]: In this test, Ag and Ab are filled in adjacent wells and are moved through gel with the help of electric current. Here, the current moves all Ags towards all Abs moving

in opposite direction. Precipitation line is formed when Ag and Ab meet in optimum proportion (Fig. 6.4.). This test is used for detection of various Ags in various body fluids ; in the diagnosis of bacterial diseases such as pneumococcal, meningococcal, *C. Diphtheriae* and *Brucella* infections ; in the diagnosis of fungal diseases such as candidiasis, cryptococcosis, histoplasmosis, etc.; in the diagnosis of viral infections such as smallpox, HBV, influenza, CMV, etc.; in the diagnosis of parasitic diseases such as amoebiasis, malaria, ascariasis, hydatid disease, etc.

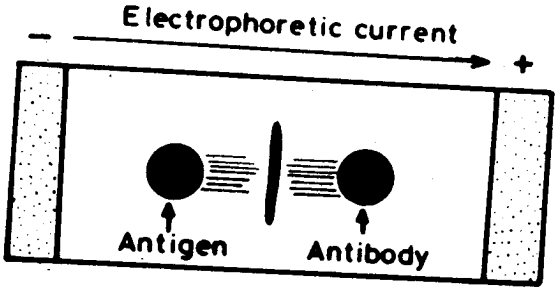

Fig. 6.4. Counter current immunoelectrophoresis

g) Rocket electrophoresis (One dimensional single electroimmunodiffusion): In this test, the Ab is mixed with agarose gel and poured on slide or plate. Wells are punched on cathodal side and charged with known and unknown Ags and electrophoresis is carried out. This results into the formation of rocket. Based on the height of rocket, the concentration of Ag is quantitated (Fig. 6.5). This test is used in quantitation of proteins and other Ags in various clinical specimens.

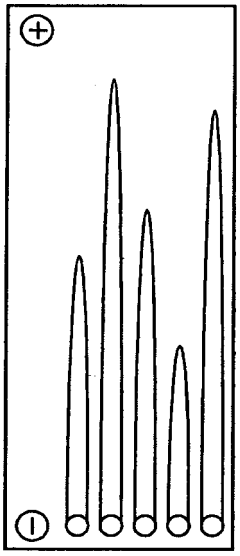

Fig. 6.5. Rocket gel Electrophoresis

h) Two dimensional immunoelectrophoresis (Laurell's procedure): This test is carried out in two steps. First step (first eletrophoresis)--to separate Ags from the well in the different parts of gel. Second step (second electrophoresis)--the Ab incorporated in agarose is poured on plate and second electrophoresis is carried out at right angle to first electrophoresis. This causes migration of Ags into stationary Abs and results in formation of cones of precipitate. Each cone indicates one antigen. This test is used to detect and quantitative human serum proteins and proteins abnormally present in various body fluids.

6.2.2. Agglutination Reaction

When a particulate Ag (agglutinogen) combines with its Ab(agglutinin) in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated. Thus, agglutination is the aggregation of already insoluble particles or cells into larger clumps.

Agglutinogen	+ Agglutinin	→ Agglutination
(Particulate Ag)	(Ab)	(Ag-Ab complex)

Mechanism of agglutination is same as that of precipitation reaction. Among the immunoglobulins, IgM with 10 binding sites is a much more powerful agglutinator than IgG with only one binding site. These IgG antibodies are unable to agglutinate due to their monovalency and such antibodies are called incomplete antibodies. The incomplete Abs are able to combine with Ag but unable to cross-link the cells and thus may act as blocking Abs and inhibit agglutination by the complete Abs. This phenomenon is known as prozone phenomenon. Agglutination can be practiced in tubes or on slides and can be visualized with the naked eye or under the microscope.

Requirements: The particulate Ag. Serum containing agglutinin (Abs). Electrolytes in the form of normal saline.

6.2.2.1. Types of agglutination reactions

1. Slide agglutination test: In this test, a smooth and uniform suspension of particulate Ag is prepared in a drop of saline on a glass slide or a tile. Then a drop of appropriate antiserum is added and mixed with a wire loop. A positive result, in few seconds, is indicated by the clumping together of the particles and clearing of drop. Clumping is visible to the naked eye but sometimes requires confirmation under the microscope. On the same slide, control test is performed in which Ag suspension is taken in a drop of saline without antiserum. If no clumping occurs, it shows that antigen is not autoagglutinable. **This test is used for** identification of bacterial isolates from clinical specimens ; blood grouping and cross matching.

The advantages are only smaller quantities of reagents are required than tube agglutination method. And the **disadvantage is -** less quantitative than tube agglutination.

2. Tube agglutination test: This is a standard quantitative test for determination of Ab titer. A fixed volume of particulate Ag suspension is added to the equal volume of

serially diluted serum in test tubes and incubated. The highest dilution of serum that gives positive agglutination reaction is recorded as Ab titer.

Uses: Used for the diagnosis of Typhoid fever (Widal test). Brucellosis (Brucella agglutination test). Typhus fever (Weil-Felix test).

Advantages: More quantitative than slide agglutination test. **Disadvantages:** Requires larger quantities of reagents.

3. Haemagglutination: Agglutination tests in which red blood cells are used are known as haemagglutination tests. The different types of haemagglutination tests are:

a) Direct active haemagglutination test: In this test, RBCs are used as an Ag.

Examples: Paul-Bunnel test for infectious mononucleosis in which sheep RBCs are used as Ag. Cold agglutination test for primary atypical pneumonia in which human 'O' group RBCs are used as an Ag.

b) Indirect active haemagglutination test: In this test, RBCs are coated with IgG Abs and the rabbit anti-IgG Abs are added which cause agglutination of RBCs. Here, the IgG form a bridge between two RBCs and anti-IgG Abs cross link the RBCs. Example, Coomb's test. Some Abs fail to agglutinate corresponding Ag and also inhibit agglutination. These are known as incomplete or blocking Abs. For example, some anti - D Abs fail to agglutinate Rh-D positive red cells. These incomplete Abs are detected by Coomb's test, which is of two types - direct and Indirect (antiglobulin test).

I. Direct Coomb's test: It is used to detect monovalent maternal Ab already present on RBCs. In this test, the sensitization of RBCs with incomplete Abs takes place in vivo. When such RBCs are treated with Coomb's serum (rabbit antiserum against human γ-globulin), agglutination occurs.

Uses: In the haemolytic diseases of newborn due to Rh incompatibility.

II. Indirect Coomb's test: The direct Coomb's test is frequently negative in haemolytic diseases due to A,B,O incompatibility. The amount of Ab bound to cell is too small for detection by direct Coomb's test. In such cases, indirect coomb's test is used for detection of Abs in the patient's serum. In this test, sensitisation of RBC with Ab is performed in vitro by incubating patient's serum with Rh positive RBCs (group 'O' or same group RBCs) and then Coomb's serum is added. This results into agglutination.

Uses: The test is used for detection of anti-Rh Ab (free) in the patients serum. Also for demonstration of any type of incomplete or non-agglutinating Abs, for example, non-agglutinating Abs in brucellosis.

c) Direct passive haemagglutination test: In this text, the Ag is adsorbed or attached to the surface of RBC. Here, RBC acts as an inert carrier of Ag. These are agglutinated by Abs.

Uses: Tanned cell hemagglutination used for the demonstration of Abs to thyroglobulin. *Treponema pallidum* haemagglutination test used for the serodiagnosis of syphilis.

d) Indirect passive haemagglutination test: In this test, sheep RBCs are sensitised with rabbit anti-sheep erythrocyte Ab (amboceptor) and used as Ag. The example is the Rose-Waaler test for rheumatoid arthritis in which the autoantibody (RA-factor) that appears in the serum acts as an Ab and agglutinates SRBCs sensitized with amboceptor.

4. Passive agglutination with latex and other particles: In this test, inert particles are used a carrier of Ag instead of RBCs. An Ag can be adsorbed on particles of bentonite or other particles of mineral origin such as polystyrene latex particles. Ag molecules are nonspecifically adsorbed to the surface of latex particles, which have uniform diameter of 0.8 - 1 μ. Addition of specific antibody transforms the latex (milk) from a milky white

liquid to a coarse suspension of visible granules. The test can be performed in the test tube but more commonly on slides.

Examples: RA factor test. Pregnancy test. CRP test (C-reactive protein). Antistreptolysin O test (ASO). The test can also be used for diagnosis of Cryptococcal meningitis. Amoebiasis. Meningococcal infections. Pneumococcal infections. *H. influenzae* infections. Hepatitis B virus infection and others.

6.2.3. Complement Fixation Test (CFT)

The ability of Ag-Ab complexes to fix complement is used to CFT. It is a versatile and sensitive test, applicable with a variety of Ags and Abs and capable of detecting as little as 0.04 μg of Ab nitrogen and 0.1 μg of Ag. Described by Bordet and Gengou (1901).

Requirements: Complement – it is obtained from guinea pig serum. The recently obtained complement is not suitable for the test, so the blood from guinea pig should be collected 12-18 hr before the test. As it is heat labile and unstable, it deteriorates at ordinary temperature therefore it should be preserved either in lyophilized or freeze dried state or by adding preservatives (equal volume of 12% sodium acetate and 4% acetic acid). Patient's serum - five ml of blood is collected from a patient in a sterile container. Serum is separated and heated at 56 ^{o}C for 30 minutes to destroy the complement present. Ag - suitable, soluble or particulate Ag obtained from commercial source or prepared in laboratory. Sheep red blood cells - defibrinated sheep blood is collected. Blood is washed in saline till supernatant is colourless. Resuspended in saline. Amboceptor (Rabbit or horse anti-sheep RBC serum). Rabbit or horse is immunized with sheep RBC's, so that it forms Abs against sheep RBCs. These Abs are called amboceptor's.

This test include two steps

Step 1: The inactivated patient's serum is incubated with its Ag in the presence of fixed amount of complement at 37 ^{o}C for 60 minutes.

Step 2: Sensitized RBCs (sheep erythrocytes coated with four MHD haemolysin) are added, mixed, and incubated at 37 ^{o}C for 30 minutes.

Results are interpreted on the basis of presence or absence of haemolysis. The test is considered as positive if there is no haemolysis. In positive test, the patient's serum contains Abs, which react with Ags and fix complement. Thus, complement is utilized in first step and there is no free complement present for lysis of SRBCs. The test is considered as negative, if there is haemolysis. In negative test, the serum does not contain Abs hence there will be no Ag-Ab reaction and complement will not be utilized in first step. It will be left intact which reacts with SRBCs and causes haemolysis. The positive result (no hemolytic) indicates presence of Abs in patient's serum. The negative result (hemolytic of Serbs) indicates absence of Abs in patient's serum (Fig. 6.6)

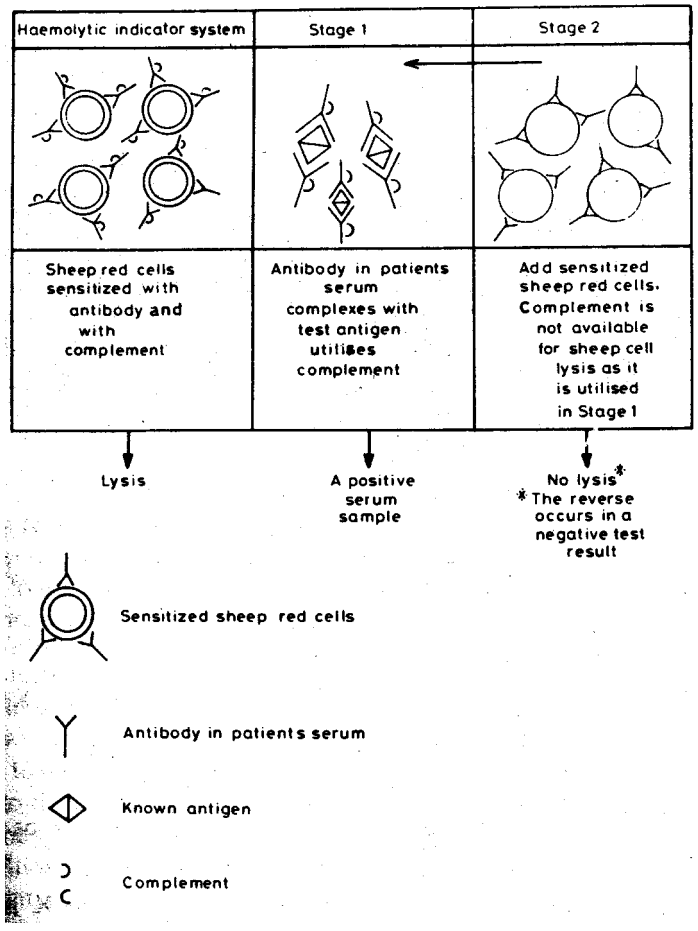

Fig 6.6 Complement fixation Test

a) Indirect complement fixation test (Indirect CFT): Certain avian (duck, turkey, parrot) and mammalian (horse, cat) sera do not fix guinea pig complement. In such cases, indirect CFT is used. The test is carried out as follows - In first step, Ag , test sera and complement are added to each other . To this a standard antiserum known to fix complement is added after first step and finally sheep RBCs with Abs are added.

In this test, If test serum contains Abs, they react with Ag hence standard antiserum will not react with Ag and complement will not be fixed. Thus, the complement causes haemolysis, which is considered as positive test. If test serum does not contain Abs, the standard antiserum will react with Ag and complement will be fixed, so that there will be no haemolysis which indicates negative test.

Ag + Test + C + Standard + SRBCs + Abs
Serum Antiserum
↓
Haemolysis - Positive test
No haemolysis - Negative test

6.2.4. Neutralization Reactions

Homologous Abs are able to neutralize the biological effects of viruses, toxins and enzymes. Such Abs are known as neutralizing Abs and the test is known as neutralization test.

a) Virus neutralization test: Virus neutralization can be demonstrated in various systems such as animals, tissue culture and chick embryos. The inoculation of virus in these systems results in multiplication and growth. Inhibition of multiplication and growth by injecting or incorporating specific neutralizing Abs indicates positive neutralization test .

The neutralization of bacteriophages can be demonstrated on a lawn culture of susceptible bacteria. When a phage is applied on the lawn culture, it causes lysis of bacteria and forms plaques but when specific antiphage antiserum is added to lawn culture, formation of plaque is inhibited because of neutralization.

b) Toxin neutralization: When antitoxin combines with toxin, it neutralizes biological effects of toxin making it harmless. Toxin neutralization can be studied in vivo and in vitro.

i) Neutralization in vivo: Schick test to determine immunity or susceptibility to diphtheria is an example of in vivo test. In this test, when diphtheria toxin is injected intradermally in a human, no reaction will occur at the site of injection, if the individual possesses circulating antitoxin in his blood. When no reaction occurs, an individual is considered as immune. Other examples of in vivo neutralization tests are - Toxigenicity testing of *C. diphtheriae* in experimental animals ; and *Cl. welhii* toxin neutralization test in guinea pig or mice.

ii) Neutralization in vitro : Antistreptolysin O test: In this test, antitoxin present in patient's serum neutralizes streptolysin O and inhibits its haemolytic activity.

6.2.5. Enzyme linked immunosorbent assay (ELISA): It is a type of binding assay that depends on Ag - Ab reaction as base and enzyme reaction as marker. This is a simple, versatile and highly sensitive test and needs only micro-liter quantities of test reagents. It is widely used for detection of variety of Abs and Ags.

Requirements :

An absorbing material specific for the Ag or Ab, such as cellulose or agarose or a solid phase such as polystyrene, polyvinyl or polycarbonate tubes or microwells or membranes or discs of polyacrylamide, paper or plastic is generally used as immunosorbent. Enzymes such as alkaline phosphatase or horse radish peroxidase. Substrates such as paranitrophenyl phosphate for alkaline phosphatase or O-phenylene diamine

dihydrochloride for peroxidase are generally used. Sodium hydroxide (3 M/l). Spectrophotometer, fluorometer or pH meter are the other requirements for this test.

There are two principal techniques- 1. Double Ab test for detection of Ag (sandwich ELISA). 2. Indirect method for detection of Ab.

The test may be performed in polystyrene tubes (macro ELISA) or in polyvinyl microtitre plates (micro ELISA)

a) Direct method (Double Ab sandwich ELISA): It is used for assay of an Ag. In this test, The wells of microtitre plate are coated with specific Ab. The sample to be tested is added and incubated overnight at $4^{o}C$ or for 2 hr. at $37^{o}C$. The wells are washed and Ab labeled with an enzyme is added and incubated at $37^{o}C$ for 1 hr. After washing a suitable substrate is added and held at room temperature. Reaction is stopped after a given time, i.e., 1 hr or after the positive control shows the development of yellow colour. The reaction is stopped by adding sodium hydroxide. The enzyme activity is measured by spectrophotometric or fluorometric electrode method.

If the test sample contains specific Ag, it is fixed to the Ab coating the wells. When the enzyme labeled Ab is added subsequently, it gets fixed on Ag and the presence of enzyme activity is indicated by the development of yellow colour which indicates positive test. If the sample is negative for Ag there will be no change in colour.

Uses: Used for detection of Ags such as Rota virus Ag, Hepatitis - B virus Ag, etc. Detection of hormones. Detection of toxins. Detection of HCG (Human chorionic gonadotrophin) - pregnancy test.

b) Indirect method: It is used for detection of Abs. In this test, Ag is coated in well instead of Ab and treated with patients serum and then antihuman immunoglobulin labeled with an enzyme is added. Rest of the procedure is almost similar to sandwich ELISA.

Uses: Used for detection of Abs in various infections such as *Salmonella, Haemophilus, V. cholerae, Brucella, Treponema*, Rubella, HIV, Hepatitis-B virus, Herpes simplex virus, Cytomegalovirus. To detect anti-DNA Abs in systemic lupus erythematosus. To detect human IgE, etc.

6.2.6. Radioimmuno Assay (fig. 6.8)

A technique in which a radioactive isotope is used to detect Ags or Abs is called radioimmunoassay (RIA). It was introduced by Berson and Yallow (1960) for quatification of plasma insulin levels. Now it is used as sensitive and specific method of quantitation of any compound to which an antibody can be produced. RIA permits the measurement up to picogram (10^{-12} g) quantities.

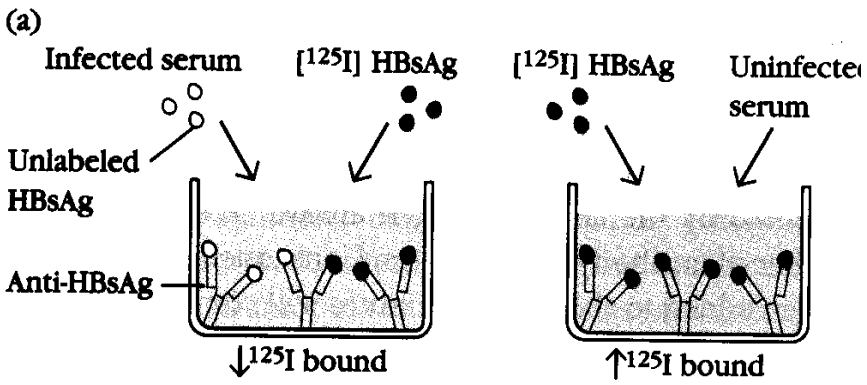

Fig. 6.8. Radioimmuno Assay

Principle : It is basically a competitive binding assay in which fixed amounts of Ab and radiolabeled Ag react in the presence of known (standard) or unknown (test) amounts of the Ag. The amounts by which the binding of labeled Ag to its Ab are competitively inhibited by increasing amounts of Ag. The amounts by which the binding of labeled Ag to its Ab are competitively inhibited by increasing amounts of standered Ag preparations are recorded. From this, a dose- response curve or standard curve is plotted. The amount of Ag present in unknown sample can be calculated from the standard curve by comparing the binding of labeled Ag.

Requirements : Antisera-of high specificity, high titre and Abs having high avidity and affinity. Labeled Ag - of high specific radioactivity. Isotopes-I ^{125} or I ^{131} are generally used. Standard Ag. Buffer - pH 7.8. Test sample.

General methodology : First, antisera against Ag to be tested is raised in Guinea pig or rabbit. Then the Ag is radio-labeled. The labeled Ag is allowed to reacte with enough Ab to bind about 70% of it. Various known amounts of unlabelled Ag are added to allow competition for Ab. After an appropriate incubation, the labeled Ag bound to Ab is separated from unbound labeled Ag by electrophoresis, chromatography, gel diffusion or double Ab method. From amounts of labeled Ag bound at various concentrations of unlabelled Ag, a standard curve is constructed, which allows quatitation of unknown Ag in test sample.

I^{125} labeled Ag + Unlabelled Ag + Ab

(Fixed) (Variable) (Fixed)

Allow to react

↓

Separate free labeled Ag from labeled Ag bound to Ab

↓

Count free and bound Ag

Applications of RIA : It can be used for quantitation for hormones, drugs, tumor markers, Ig E, viral Ags, autoimmune markers etc.,

6.3. Summary

Immunological reactions are mainly between antibody and antigens. The reactions are mainly of five types. They are precipitation, agglutination, neutralization, complement fixation, ELISA and Radio immunoassay. In precipitation reactions the soluble antigens

combine with its antibody and form an insoluble precipitate. Where as in agglutination reactions the particulate antigen reacts with its antiboby and form into clumps (get agglutinated).In complement fixation reactions, fixation of complement occur during the interaction between Ag and Ab. Thus, in this teat, the consumption of complement in-vitro can be used as a test and measure Ab, Ag or both. The ability of homologous Abs to neutralize the biological activities of viruses, toxins and enzymes is used as a principle in Neutralization reactions. In ELISA, the Abs linked with enzymes were used, which give characteristic colour on addition of suitable substrate, to react with Ags. RIA is the competitive binding assay where the amounts by which the binding of labeled Ag to its Ab are competitively inhibited by increasing amounts of standard Ag preparations are recorded.

6.4. Model Questions

Write an essay on different types of precipitation and agglutination reactions

Write in detail about the complement fixation and Radio immuno assay.

Write short notes on the following

a) Precipitation tests
b) Agglutination reactions
c) Complement fixation test
d) Neutralization test
e) ELISA
f) RIA

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LESSON NO 7.

HYPERSENSITIVITY

7.0. Objectives : Both Immediate and delayed types of Hypersensitivity reactions were discussed in detail in this lesson.

7.1. Introduction

Immune response, usually beneficial to the host, may sometimes become harmful also. Sensitized individuals may respond to subsequent antigenic stimuli in heightened or exaggerated manner leading to Tissue damage, Disease, or even death of the individual. This type of response is termed as hypersensitivity.

The term 'allergy' was coined by Von Pirquet (1906) to cover any altered response to an antigen. Therefore, increased resistance is immunity and increased susceptibility is hypersensitivity. The terms allergy and hypersensitivity are now used synonymously for altered state of the body induced by an antigen.

In allergic (hypersensitivity) reactions, An antigen - referred as allergen or sensitiser. Immunization – referred as sensitization. An individual - referred as hypersensitive or allergic.

Classification:

Based on the time required for a sensitized host to develop clinical reactions upon re-exposure to the Ag, hypersensitivity reactions are classified into two types - Immediate hypersensitivity and Delayed hypersensitivity.

Immediate type of hypersensitivity: Hypersensitive state in which an allergic reaction develops immediately within a short period after contact with an Ag (sometimes within a few seconds also) is called immediate type. It is a B-cell or Ab mediated reaction, examples are Anaphylaxis, Ab mediated cytotoxic hypersensitivity, and Ag-Ab complex mediated hypersensitivity.

Delayed type of hypersensitivity: Hypersensitive state in which there is an appreciable delay between the exposure to the Ag and development of symptoms is referred as delayed type. It is a T-cell mediated reaction, e.g., Infection (tuberculin) type and Contact dermatitis type.

Table 7.1 Differences between Immediate and delayed Types of Hypersensitivity

Immediate	**Delayed**
1. Appears immediately within short time and recedes rapidly.	Appears slowly in 24 – 72 hr and lasts longer
2. Induced by Ag or hapten by any route	Induced by infection, injection of Ag or hapten intradermally with Freund's adjuvant or by skin contact
3. Antibody mediated reaction	Cell mediated reaction
4. Passive transfer possible by serum	Passive transfer possible with lymphocyte or transfer factor
5. Desensitisation easy but short lived	Desensitisation difficult but longer lasting

Coombs and Gel Classification: Coombs and Gel in 1963 classified hypersensitivity reactions into four types based on different mechanisms of pathogenesis. This classification is widely accepted. The types are: Type I - anaphylaxis (IgE or reagin dependent). Type II - Ab mediated cytotoxic hypersensitivity. Type III - immune complex mediated hypersensitivity. Type IV -Delayed or cell mediated hypersensitivity. One additional type –type V, has been recently proposed. Type I, II, and III are Ab mediated reactions. Type IV is cell mediated reaction.

7.2. Type I---Antibody mediated Anaphylaxis (IgE or reagin dependent)

Introduction: It is an IgE mediated immediate type of hypersensitivity reaction. The term anaphylaxis (ana-without, phylaxis-protection) was coined by Richet in 1902 to describe his experiment on dog. Theobald Smith (1902) had noticed a similar phenomenon in guinea pigs following injections of toxin-antitoxin mixtures. When a guinea pig is injected with a small does of an antigen (about 1 mg) such as egg albumin, no adverse effects are noticed. When a second dose of same antigen (i.e. egg albumin) is injected intravenously after an interval of 2-3 weeks, the sensitised guinea pig reacts very dramatically and a condition known as 'anaphylactic shock' is developed. The animal becomes restless, cyanosed, may develop convulsions and die. The initial injection of antigen is termed as sensitising dose. It is more effective by parenteral route. The second injection of antigen is termed as shocking dose. It is more effective by intravenous route than intraperitoneal or subcutaneous route. During an interval between the two injections, the animal forms antibodies. Anaphylaxis is the result of interaction of the shocking dose of antigen with newly formed antibodies on the surface of tissue cells. This interaction triggers the release of pharmacologically active substances, which increase capillary permeability and cause smooth muscle contraction. Guinea pigs are highly susceptible to this reaction. Rabbit, dog and human beings show intermediate susceptibility while rats are resistant.

7.2.1. Mechanism

7.2.1.1. Types of cells involved in anaphylaxis: The cell types of greatest importance in the production of anaphylactic reaction are the mast cells. They are normally present in submucosal layers of respiratory tract, gastrointestinal tract, skin and vascular endothelium. Basophils of blood also participate in this reaction.

7.2.1.2. Antibody involved in anaphylaxis: Reaginic antibodies possessing specific configuration, which is complementary fit for specific receptor size on tissue cell and having a strong affinity for tissue cells are responsible for anaphylaxis. These antibodies belong to the class IgE. The IgG antibodies can also act as reagins in the guinea pig and mouse. The contribution of IgG in human anaphylaxis is not known.

7.2.1.3. Steps involved in the mechanism of anaphylaxis: The first step is the synthesis of antibodies capable of binding to mast cells and basophils. For this, an antibody must possess a specific configuration, which is complementary fit for specific receptor site known a Fc ER (Fc receptor of IgE) on mast cells and basophils. The IgE antibodies coat the mast cells and basophils with the help of Fc region (CH-3 and CH-4 domains) and bind specifically to FcER sites on the mast cell surface. The second step in anaphylaxis is the combination of the cell fixed antibodies (Fab fractions) with specific antigen of shocking dose, bridging the gap between adjacent antibody molecules on cell. This interaction increase the permeability of the cells to calcium ions and cause degranulation through proteolytic enzymes, which leads to the release of pharmacologically active substances present in the granules (Fig. 7.1).

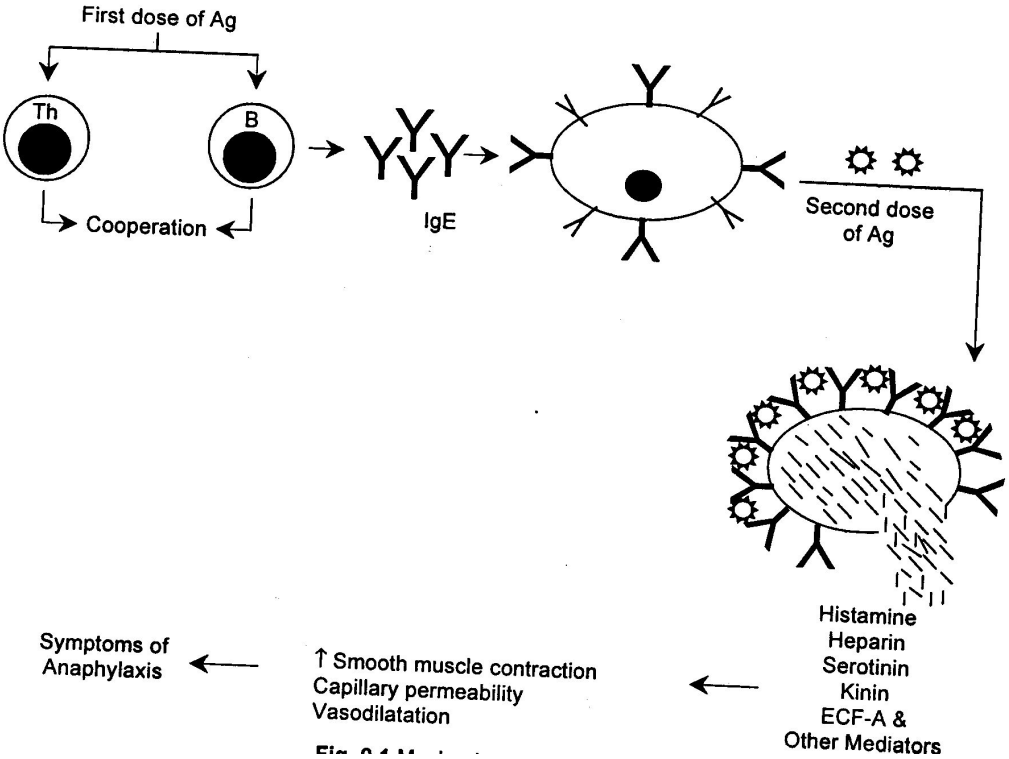


Fig. 7.1.Mechanism of Anaphylaxis

7.2.2. Pharmacological mediators of anaphylaxis: There are two types of mediators

Primary mediators are the preformed contents of mast cell and basophil granules. These include histamine, serotonin, eosinophil chemotactic factor of anaphylaxis, neutrophil chemotactic factor of anaphylaxis, heparin and various proteolytic enzymes.

Secondary mediators are newly formed upon stimulation by mast cells, basophils and other leucocytes. These include slow reactive substance of anaphylaxis, prostaglandins and platelet activating factors.

Other mediators: These include the anaphylatoxin released by complement activation and kinins formed from kininogen present in plasma (plasma globulins). These are other biologically active substances in addition to the mediators released by mast cells and other leucocytes.

7.2.2.1. Nature and functions of Primary mediators

i. Histamine: Formed by decarboxylation of histidine present in the granules of mast cells, basophils and platelets. Induces smooth muscle contraction in tissues and organs such as intestine, uterus and especially the bronchioles in man and guinea pigs. Also cause a marked increase in capillary permeability and is a potent vasodilator.

ii. Serotonin (5-hydroxy tryptamine): Formed by decarboxylation of tryptophan. Causes bronchial and ileal smooth muscle contraction in guinea pig. Also increases capillary permeability and vasoconstriction. It is main mediator of anaphylaxis in rats and mice and does not play any significant role in human beings.

iii. Heparin: An acidic mucopolysaccharide stored in mast cells and basophils. It can block complement cascade, inhibit coagulation and fibrinolysis. Contributes to anaphylactic reaction in dogs, but apparently not in human beings.

iv. Eosinophil chemotactic factors of anaphylaxis (ECFA): Acidic tetrapeptide released by mast cells and basophils. Chemotactic for eosinophils - probably contribute to the eosinophilia. Also enhance C3b activity and cause antibody and complement dependent damage to some parasites.

7.2.2.2. Nature and functions of Secondary mediators

i. Slow reacting substance of anaphylaxis (SRS-A): An acidic lipid (leukotrienes). Acts on smooth muscles of large blood vessels and bronchi. It is more potent bronchoconstrictor than histamine. Its action is slow, prolonged and is not inhibited by antihistamines. Predominant pharmacological mediator in human asthma.

ii. Prostaglandins and thromboxanes: Derived from arachidonic acid formed from disrupted cell membranes of mast cell and other leucocytes. Prostaglandin F2a and thromboxane are transient bronchoconstrictors. Prostaglandin E2 is a bronchodilator.

iii. Platelet activating factor (PAF): Lipid generated and released by basophils and mast cells. Causes aggregation of platelets and release of preformed serotonin (vaso active amine).

7.2.2.3. Nature and functions of other mediators

i. Kinins: Polypeptides produced by the action of proteolytic enzymes on kininogen. Cause smooth muscle contraction, increased vascular permeability, vasodilatation and pain. The best known kinin is bradykinin. Its role in human anaphylaxis is not known.

7.2.3. Types of anaphylaxis

7.2.3.1. Systemic anaphylaxis: This is a condition of an acute shock usually terminating in death following the injection of an antigen into a previously sensitised

animal. Death occurs due to suffocation from contraction of muscles in the walls of bronchioles.

7.2.3.2. Passive systemic anaphylaxis: The animal can be passively sensitised to systemic anaphylaxis by injecting serum from another already sensitised animal and then the animal can be shocked by antigen that is used for sensitisation. The symptoms are same like systemic anaphylaxis.

Systemic anaphylaxis is man: Systemic anaphylaxis in man is rare. Man develops systemic anaphylaxis following - Insect bite such as bee or wasp stings ; Antitoxic serum such as antitetanus (ATS), antidiphtheritic (ADS) or antigasgangrene serum (AGS) and Antibiotics such as pencillin.

Clinical features: Lung is the principal shock organ in man hence the symptoms are - severe respiratory distress due to bronchiolar construction with vascular collapse ; Laryngeal oedema ; Acute hypotension ; Loss of consciousness and finally death.

7.2.3.3. Cutaneous anaphylaxis (Local anaphylaxis): By intra-dermal injection of antigen into actively sensitized host, a local wheal and flare reaction may be produced. The wheal is a pale, central area of puffiness due to oedema, which is surrounded by a flare caused by hyperaemia and subsequent erythema. The reaction can be used as skin test for testing hypersensitivity and to identify the allergen responsible for atopy. (In highly sensitised individuals, the skin test may lead to systemic anaphylaxis terminating in death, hance the test should be carried out by taking all precautions).

Passive cutaneous anaphylaxis (PCA): The cutaneous anaphylaxis may also be induced passively by the intra-dermal injection of antibody and the local wheal and flare reaction may then be demonstrated by intravenous injection of antigen with dye such as Evan's blue after 4-24 hr. Evan's blue is able to combine with serum protein. At the sensitised site, the combination of antigen with antibody increases capillary permeability and vasodilatation and permits the escape of protein dye complex at the site of intra-

dermal injection that results in a immediate bluing. PCA can be used as an extremely sensitive in vivo method for detection of antibodies. PCA reaction conducted on human being is known as Prausnitz-Kustner (PK) reaction.

Local anaphylaxis in man (Atopy): Hypersensitive states in which a person reacts with substances encountered during the course of everyday life are known as atopy (meaning out of place or strangeness) or idiosyncrasies. The term atopy was coined by Coca (1923). About 10% of the population suffer from atopy to allergens such as grass pollen, animal danders, mites in house, dust, food, etc.(allergens). Contact of allergen with cell bound IgE in the bronchial tree, the nasal mucosa, the conjunctival tissues, intestine or skin releases pharmacologically active mediators and produces symptoms of Asthma, Hay fever (allergic rhinitis), Conjunctivities, Gastrointestinal symptoms, Dermatitis, Urticaria in persons allergic to food such as strawberry.

Reactions occur at the site of entry of the Ag, e.g., inhalation of allergen affects lungs, leading to asthma but asthmatic symptoms may also be caused by the ingestion of the allergen, which is then carried to the respiratory tract via blood.

Mechanism: The Abs responsible for atopy are known as reagins. These are IgE type of antibodies, heat labile, inactivated at $56^{o}C$ in 2-4 hr. Atopic sensitivity is due to excessive production of IgE antibodies and often associated with a deficiency of IgA. The lymphocytes responsible for synthesis of IgA and IgE are parallely distributed in the respiratory and intestinal submucosa. In normal individuals, the antigen interacts with IgA producing lymphocytes and hence the IgE producing lymphocytes do not come in contact of antigens. When IgA is deficient, an Ag causes massive stimulation of IgE producing lymphocytes, leading to overproduction of reagins responsible for the clinical expression of atopic reactions.

7.2.4. Desensitisation

Acute or temporary desensitisation: It is a specific desensitisation with the help of a series of carefully graded injections of the serum beginning with minute doses for 6-8 hr, then the full dose can be administrated without danger. This desensitisation is short lasting.

Prolonged desensitisation: Longer lasting desensitisation can be achieved by giving repeated injections of antigen weekly or biweekly in increasing amounts. This results in production of blocking antibodies (IgG), which combine with allergens and prevent their contact with cell fixed IgE. An alternative method is an injection of allergen with oil adjuvant (depot therapy). In this case, desensitisation occurs due to continuous production of IgG, which is the result of slow and continuous release of allergen from injection site.

Anaphylaxis in vitro: Isolated tissues such as uterus or ileum from sensitized guinea pigs, held in a bath of ringer solution or isotonic fluid will contract vigorously on addition of the specific antigen. This is known as Shultz-Dale phenomenon. Tissues from normal animals can be passively sensitized by treatment with serum from sensitized animal.

Anaphylactoid Reaction: It is a type of reaction that clinically resembles anaphylactic shock. It develops following the intravenous injection of peptone, trypsin, heavy metal salts, starch, or polysaccharide. Its clinical resemblance is due to the same chemical mediators participating in both the reactions. This reaction has no immunological basis and occurs non-specifically due to the activation of the alternative complement pathway and release of anaphylatoxin.

7.3. Type II--Cytotoxic Reaction: Reactions of this type are initiated by antigenic component, which is either part of tissue cell or closely associated with a cell, e.g., microbial product or a drug attached to cell wall. Combination of this Ag with IgG or IgM Ab damages that cell by promoting contact with phagocytic cells either by -

reduction in surface charges or by opsonic adherence directly through Fc or by immune adherence through bound C3. Cell death may also occur through activation of full complement pathway up to C8 and C9 (Fig. 7.2).

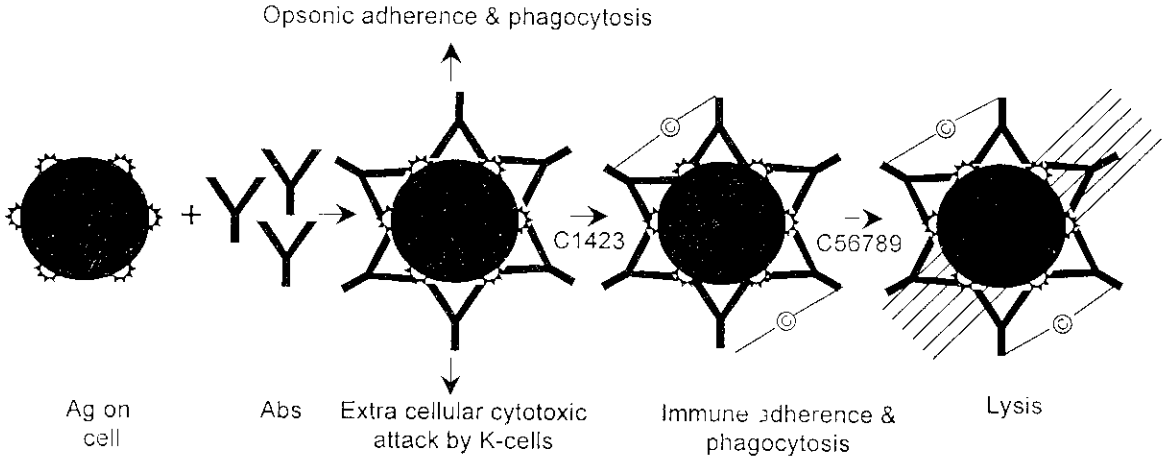
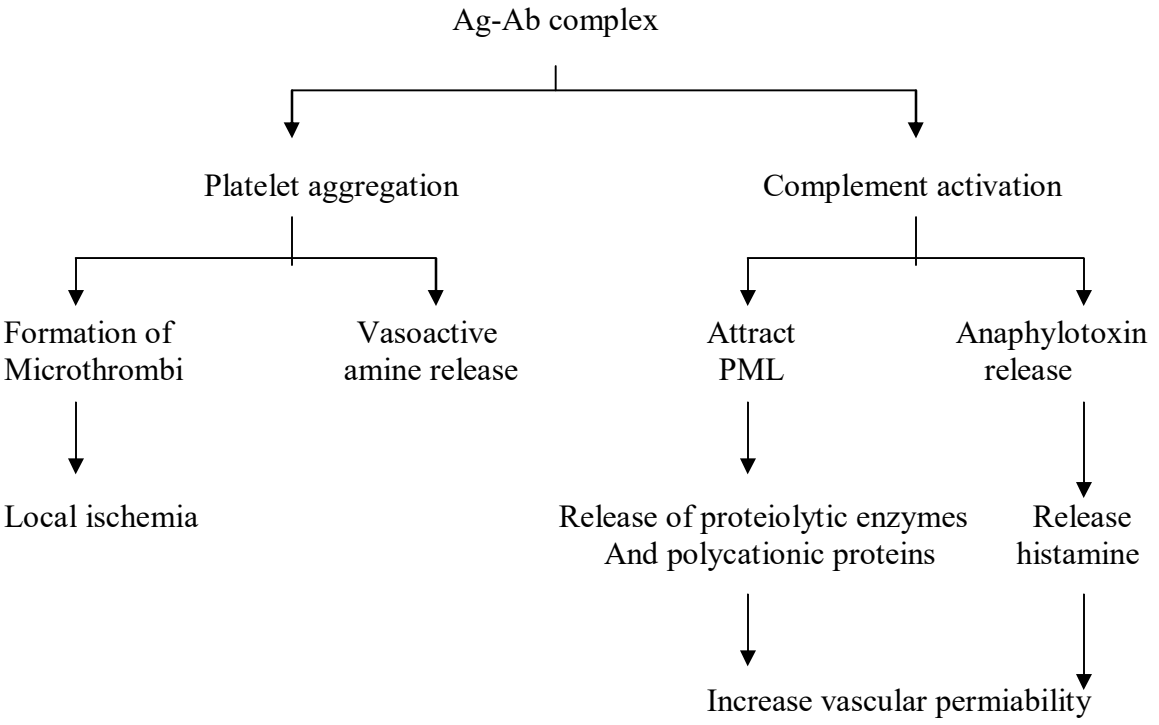


Fig. 7.2. Mechanism of cytotoxic reaction

Examples: Isoimmune reactions such as ABO transfusion reactions, erythroblastosis foetalis. Autoimmune Reactions, Lysis of RBCs caused by antierythrocyte Abs in autoimmune anaemias. Lysis of platelets by autoantibodies against platelets in autoimmune thrombocytopenia. Lysis of neutrophils by autoantibodies in agranulocytosis. Drug reactions, Drugs such as penicillin, phenacetin, quinidine, sedormid, sulfonamides, thiazide, chlorpropamide attach to the surface of cells such as RBCs, neutrophils or platelets causing alterations in the surface antigenicity that initiate Ab synthesis. Combination of these Abs leads to Cytotoxic or Cytolytic reactions. Classical example is thrombocytopenic purpura produced by sedormid (sedormid purpura). In certain bacterial reactions such as *Salmonella* and mycobacterial infections - their products coat the surface of RBCs and induce Ab synthesis and subsequent infection by same or related pathogen cause haemolysis.

7.4. Type III: Immune Complex Mediated Hypersensitivity: It is a type of antibody mediated hypersensitivity reaction characterized by deposition of Ag-Ab complexes in tissues particularly on endothelial surfaces. The Ag-Ab complex formation

in and around small blood vessels may result in acute inflammatory reactions and sometimes mechanical blockage of the vessels causing interference with blood supply to surrounding tissues. Ag-Ab complex may activate complement. If activated, complement will cause release of anaphylatoxin that causes histamine release with vascular permeability changes. Activation of complement also results in aggregation of polymorphonuclear leucocytes (PML), which start phagocytosis of the immune complexes, this in turn causes the release of proteolytic enzymes and polycationic proteins from the granules of PML, which increase from the granules of PML, which increase capillary permeability (Flow chart). Activation of complement and massive infiltration by PML and attraction of platelets lead to inflammation and tissue injury.



Flow chart of Type III hypersensitivity

There are of mainly two types of type III reactions - Arthus reaction, and Serum sickness.

7.4.1. Arthus reaction: It is an inflammatory reaction with particular involvement of blood vessels, which may occur in the tissues of sensitised animals as a result of local Ag-Ab reaction. It is a type III reaction, first described by Arthus in 1903. He observed that repeated subcutaneous injections of soluble Ag (horse serum) into rabbits produced high level of precipitating Abs (hyperimmunization). Injection of same Ag intradermally or subcutaneously into hyperimmunized rabbit produced an erythema, oedema, induration and haemorrhagic necrosis, which reaches a peak in 3-6 hr. This type of reaction is known as Arthus reaction.

Mechanism: It is a local reaction that occurs at local site, i.e., in and around the walls of small blood vessels. Reaction occurs when an appreciable level of Abs, mainly IgG type, are produced by hyperimmunization. IgG reacts with Ag - forms complex .

Ag-Ab complexes diffuse into vessel walls

↓

Activate complement

↓

Chemotactic complement components attract neutrophils and induce intravascular clumping of platelets.

↓

Neutrophils ingest complexes and release lysosomal enzymes, which damage neighboring cells and cause necrosis and inflammatory reaction.

↓

Aggregation of platelets causes vascular occlusions leading to ischemic necrosis of blood vessels.

Arthus reaction—special feature: Activation of Complement by classical or alternative pathway is must for Arthus reaction. Arthus reaction can be passively transferred with sera containing Abs. In man, Arthus like reaction is produced in individuals who have received several injections of antitoxic sera an insulin.

7.4.2. Serum sickness: It is a systemic form of type III reaction. The term serum sickness was first applied by Von Pirquet and Schick in 1905 to a condition that may occur in man following the injection of horse serum and sometimes following the injection of drugs such as sulfonamide, penicillin, streptomycin and organic arsenicals. The drugs mentioned above are not antigenic but form antigenic complexes by combination with plasma or tissue proteins. A single dose of Ag is sufficient to produce the reaction. It can serve both as sensitising dose as well as shocking dose. The symptoms appear after 7-14 days following a single injection of a high concentration of foreign serum such as diphtheria antitoxin or antitetanus serum.

The symptoms are: Fever, Lymphadenopathy, Splenomegaly, Arthritis, Glomerulonephritis, Endocarditis, Vasculitis, Urticaria, Abdominal pain, Nausea and vomiting.

The Pathogenesis - is the formation of immune complexes, which get deposited on endothelial lining of blood vessels at the site of lesions in various parts of the body causing inflammatory infiltration. The damage to tissue produced is same as in Arthus reaction. The disease is self limited. With continued rise in Ab production, Ag-Ab complexes become larger in size and more susceptible to phagocytosis and immune elimination.

7.5. Type IV - Delayed Hypersensitivity: This term is applied to a group of hypersensitivities in which there is an appreciable delay between the exposure to Ag and the development of symptoms. It may be defined as an increased reactivity to specific Ags mediated by T-cells and not by Abs. Delayed hypersensitivity is demonstrated by a cutaneous reactivity, which usually becomes visible after 24-48 hrs following introduction of Ag. The cutaneous reactions are inflammatory and indurated type involving lymphocytes and macrophages and not wheal and flare type as seen in anaphylaxis. The reaction is inducted by sensitised T-cells which, on contact with the specific Ag, release lymphokines that cause biological effects on leucocytes, macrophages, and tissue cells. Passive transfer-delayed hypersensitivity is a cellular

phenomenon hence cannot be transferred passively by serum. Passive transfer to a normal recipient is possible with the help of sensitised T-lymphocytes from a sensitised donor or with the help of transfer factor. It occurs in two main clinical forms. The tuberculin (infection) type. The contact dermatitis type.

7.5.1. Tuberculin (infection) type: It develops as a result of infection with tubercle bacillus and is demonstrated by tuberculin reaction. When a small dose of Ag (tuberculin) 1-5 TU (1 TU, equivalent to 0.01 mg OT or 0.00002 mg PPD) is injected intradermally in an individual sensitised to tubercular protein by previous infection or immunization, an erythema and indurated swelling develop gradually, which reach maximal intensity and size after 24-72 hr and then slowly regress. Histologically, the reaction is characterized by an infiltration of injection site with mononuclear cells, mainly lymphocytes and 10-20% macrophages. These inflammatory cells (macrophages and lymphocytes) may be seen around blood vessels and nerves. Tuberculin type of hypersensitivity is also developed in many other infections such as leprosy, brucellosis, lymphogranuloma venereum and in most fungal, viral, and parasitic infections, especially when infection is subacute or chronic and pathogen is intracellular.

7.5.2. Contact dermatitis type: Delayed hypersensitivity, sometimes, develops as a result of exposure of skin to a variety of substances such as: Drugs like penicillin, sulfonamide, organic arsenicals, etc. Metals like nickel, cobalt, chromium, etc. Chemicals like picryl chloride, dinitrochlorobenzene, formaldehyde, iodine, dyes, cosmetics, soaps, etc. These substances are not antigens but act as haptens. After absorption in skin these substances covalently combine with skin proteins and become antigenic. Contact of these substances in a sensitised individual leads to contact dermatitis. The lesions vary from macules and papules to vesicles that breakdown leaving behind raw weeping areas typical of acute eczematous dermatitis. The cutaneous inflammation is similar to tuberculin type. Histologically, there is a mononuclear cell infiltration in the upper layers of the skin and around hair follicles. In this, it may be possible to identify the substance responsible for sensitivity by patch test in which the

suspected substance is applied to skin under an adherent dressing and observed for itching (4-5 hr) and local reaction (erythema to vesicle or blister formation in 24-28 hr).

7.6. Summary

Hypersestitivity reactions are broadly categorized into two types depending on the time taken for the reaction – namely early and delayed types. Coombs and gel classified them in to four types. Type 1 - This type of reactions mediated by Ig E molecules are referred to as allergic reactions. Type I is an immediate type of hypersensitivity reaction, involving IgE antibodies. In this type, the synthesized IgE will bind over to the mast cell and basophils and result in degranulation leading to the release of pharmacologically active substances. In type II cytotoxic reactions, IgG or IgM antibodies mediated cell death occurs through the activation of complement pathway. In type III reactions, Ag-Ab complexes will get deposited in the tissues, particularly on the endothelial surfaces. In delayed type reactions, the activated T-cells release lymphokine which activate the macrophages and results in inflammation.

7.7. Model Questions

a) Write in detail about types of anaphylactic reactions.
b) Give an account on type II and III hypersensitive reactions
c) Write an essay on type IV type of hypersensitive reactions

Write short notes on the following

a) Arthus reaction
b) Serum sickness
c) Systemic anaphylaxis
d) Primary mediators
e) Tuberculin reaction
f) Contact dermatitis

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LESSON NO. 8.

AUTOIMMUNITY

8.0. Objectives : In this lesson nature of different types of autoimmune diseases were described in detail and the therapeutic measures for these diseases was added.

8.1. Introduction

The recognition of self and non-self is an important function of immune system. Response to self as antigen is prevented by appropriate mechanisms. This is known as 'tolerance to self'. Under some circumstances, these mechanisms must break down and self antigens become recognized as foreign antigens. When this occurs, auto antibodies or sensitized lymphocytes capable of reacting with self components are produced. This abnormal response is known as **autoimmunity**. The autoimmunity may lead to autoimmune diseases. Thus, autoimmune disease is a condition in which the natural

unresponsiveness or tolerance to self terminates, as a result of which antibody or sensitized lymphocytes reacts with self antigen causing disease.

8.1.1. Criteria for autoimmune diseases

The criteria are known as Witbsky's postulates.

1. The autoimmune response must be regularly associated with disease.
2. The antigen responsible for the immune response must be identified, isolated and characterized.
3. A replica of the disease must be inducible in laboratory animals and immunopathological changes in the natural and experimental diseases should parallel t to each other.
4. Transferring autoantibody or self –reactive lymphocytes to a host should reproduce the disease.

8.1.2. Causes of autoimmune disease

8.1.2.1. Genetic susceptibility : The most common evidence for the existence of a genetic predisposition to autoimmune disease s in the higher incidence of the disease in monozygotic twins, with a lower but still increased incidence in dizygotic twins and family members compared to an unrelated population.

8.1.2.2. Hidden or sequestered antigen theory

According to this theory, during embryonic development, tissues that are exposed to the lymphoreticular system are recognized as self antigens and hence are unable to induce immune response. However, many auto antigens e.g., eye lens protein, brain tissue protein, thyroglobulin, have no opportunity to develop tolerance because they are anatomically confined to sites that prevent their access to lymphoreticular system. Though these antigens are self antigens, they are recognized as non-self. Contact of these

antigens with immunocompetent cells results in immune response leading to trauma or injury. Spermatozoa have no opportunity to develop tolerance because they develop with puberty, hence treated as non-self. Exposure of the sequestered tissue antigens through trauma or infection in later life leads to autoimmune disease.

8.1.2.3. Altered forms of self antigens or neoantigens :

In certain circumstances, the native tissue antigens may undergo antigenic alteration by physical (irradiation), chemical (drugs and other chemicals) or biological (viral infections, microbial enzymes) means and thus assumes a new antigenic specificity. The antibody formed against such antigens react with native antigens and injure the cell. Antigens may also arise by mutations.

8.1.2.4. Shared or cross reacting antigens

Some organisms carry antigenic determinants that resemble host cell components. These are the cross reacting antigens, which may induce an immune response damaging the particular organ or tissue in the host. For example, in rheumatic fever, antibodies produced against group A Streptococcus react with human heart tissue and produce injury. Another example is the neurological injury that occurs sometimes following anti-rabis immunization in human being. Anti-rabis vaccine prepared by using sheep brain tissue when injected, elicits an immune response against sheep brain antigens, which damage the host's nervous tissue due to the cross reaction between human and sheep brain antigens.

8.1.2.5. Loss of immunoregulation

Functional loss of activity of T helper cells (enhanced Th activity) and T- suppressor cells (decreased Ts activity) results in heightened antibody or T cell response. This loss is synonemous with the loss of self tolerance and self antigens behave like foreign antigens leading to autoimmune disease. Defects in the thymus in stem cell development and

macrophage function have also been considered as causes. Another hypothesis is non-specific polyclonal B-cell activation by certain stimuli such as 2-mercatoethanol, lipopolysaccharide, trypsin, nystatin and infections with some organisms like mycoplasma, EB virus (Epstein-Barr virus) and malarial parasites.

8.1.2.6. Genetic abnormalities

The mutation of immunocompetent cells to antigenic responsiveness to self antigens that results in autoimmune disease. Defects in immune response genes or immunoglobulin genes may also be the cause of autoimmune disease. A disordered immune regulation based upon genetically immune regulation based upon genetically determined imbalances of the T-helper /inducer and T suppressor/cytotoxic is an important determinant in the development of autoimmune disease as per recent evidence.

8.2. Classification of Autoimmune Diseases

Traditionally, autoimmune diseases have been classified as B-cell or T-cell mediated diseases. Autoimmune diseases are classified into four types based on the site of involvement and nature of lesions.

8.2.1. Autoimmune Hemolytic anemia

Hemolytic anemia is autoimmune when antibodies react with self red blood cells (RBCs). In this connection, the number of RBCs in the circulation is decreased because antibody directed against an antigen on the surface of the blood cell destroys of removes the cells. The destruction of the RBCs can be attribute to two mechanisms. The destruction of the RBCs can be attributed to two mechanisms. One involves the activation of the complement cascade and eventual lysis of the cells. The resultant release of hemoglobin may lead to its appearance in the urine – that is **hemoglobinuria**. The second is by the opsonization of RBCs facilitated by antibody and the C3b components of component. In

the later case, the RBCs are bound to and engulfed by macrophages whose receptors for Fc and C3b attach to the antibody –coated RBCs.

It is customary to divide the antibodies responsible for autoimmune hemolytic anemia into two groups on the basis of their physical properties. The first group consists of the warm autoantibodies, so-called since they react optimally with RBCs at 37 ^{o}C. The warm autoantibodies belong primarily to the IgG class, and some react with rhesus (Rh) antigens on the surface of the blood cells. Because activation of the complement cascade requires the close alignment of at least two molecules of IgG and Rh antigens sparsely distributed on the surface of the erythrocyte, complement-mediated lysis does not occur. On the other hand, IgG antibodies to these antigens are effective in inducing immune adherence and phagocytosis. Individuals with autoimmune hemolytic anemia can be identified by a coombs test, which is designed to detect bound IgG on the surface of RBCs.

A second kind of antibody, the cold agglutinins, attaches to RBC's only when the temperature is below 37 ^{o}C and dissociates from the cells, when the temperature rises above 37 ^{o}C. Cold agglutinins belong primarily to the IgM class and are specific for I or i antigens present on the surface of RBCs. Since the cold agglutinins belong to the IgM class, they are highly efficient at activating the complement cascade and causing lysis of the erythrocytes to which they attach. Nevertheless, haemolysis is severe in patients with autoimmune hemolytic anemia due to cold agglutinins, as long as their body temperature is maintained at 37 ^{o}C. When arms, legs, or skin are exposed to cold and the temperature of circulating blood is allowed to drop, severe attacks of haemolysis may occur.

Although the cause of autoantibody formation is often not known, some clues are offered by drug-induced anemia. A drug like penicillin, which behaves as hapten, may bind to some protein on the surface of RBCs, and this entire complex may then act as an antigen eliciting antibodies to the surface of the cell, causing lysis or phagocytosis. In such case, however, the disease is self-limited and disappears when the drug use is discontinued.

Another example of drug-induced anemia occurs in a small minority of patients using α- methyldopa, an anti-hypersensitive drug. It leads to a disorder that is almost identical to that characterized by warm autoantibodies. Sometimes cold agglutinins appear after infection by *mycoplasma pneumoniae* or viruses, implicating a role of an infectious disease trigger in genetically susceptible individuals.

8.2.2. Myasthenia Gravis

Another autoimmune disease in which antibodies to a well defined target antigen are implicated is myasthenia gravis. The target self-antigen in this disease is the acetylcholine receptor at the neuromuscular junctions. The autoantibody acts as an antagonist that blocks the binding of acetylcholine (Ach) to the receptor. This inhibits the nerve impulse from being transmitted across the neuromuscular junction, resulting in severe muscle weakness, manifested by difficulty in chewing, swallowing and breathing, and eventual death from respiratory failure. It effects individuals of any age, but the peak incidence is women in their late 20s and men in their 50s and 60s. The female to male ratio is approximately 3:2. Some babies of myasthenic mothers have transient muscle weakness, presumably because they received sufficient amounts of pathogenic IgG by transplacental passage.

The disease can be experimentally induced in animals by immunization with Ach receptors purified from torpedo fish or electrical eel, which demonstrate significant cross-reactivity with mammalian receptors. In the experimental disease, resulting from the formation of antibodies against the foreign receptors, the antibodies bind to the mammalian receptors and mimic almost exactly the natural form of the disease. The disease may be passively transferred with antibody.

The development of myasthenia gravis appears to be linked to the thymus, since many patients have concurrent thymoma, or hypertrophy of the thymus, and removal of thymus sometimes leads to regression of the disease. Molecules cross-reacting with the ACh receptor have been found on various cells in the thymus, such as thymocytes an the

epithelial cells, but whether these molecules are the primary stimulus for the development of the disease is unknown. There is a genetic component to the disease as myasthenia gravis is associated with HLA – DR3 alleles.

8.2.3. Graves Disease

One of the main manifestations of the graves disease is a hyperactive thyroid gland (Hyperthyroidism). This aspect of disease serves as an example in which antibodies directed against a hormone receptor may activate the receptor rather than interferes with its activity. For reasons not yet understood, in graves disease, patients develop autoantibodies against thyroid cell - surface receptors for thyroid-stimulating hormone (TSH). The interaction of these antibodies with receptor activates the cell in a manor similar to activation by TSH. Hence, the autoantibody behaves as an agonist. The long lasting stimulation by these antibodies causes hyperthyroidism due to the continuous stimulation of the thyroid gland.

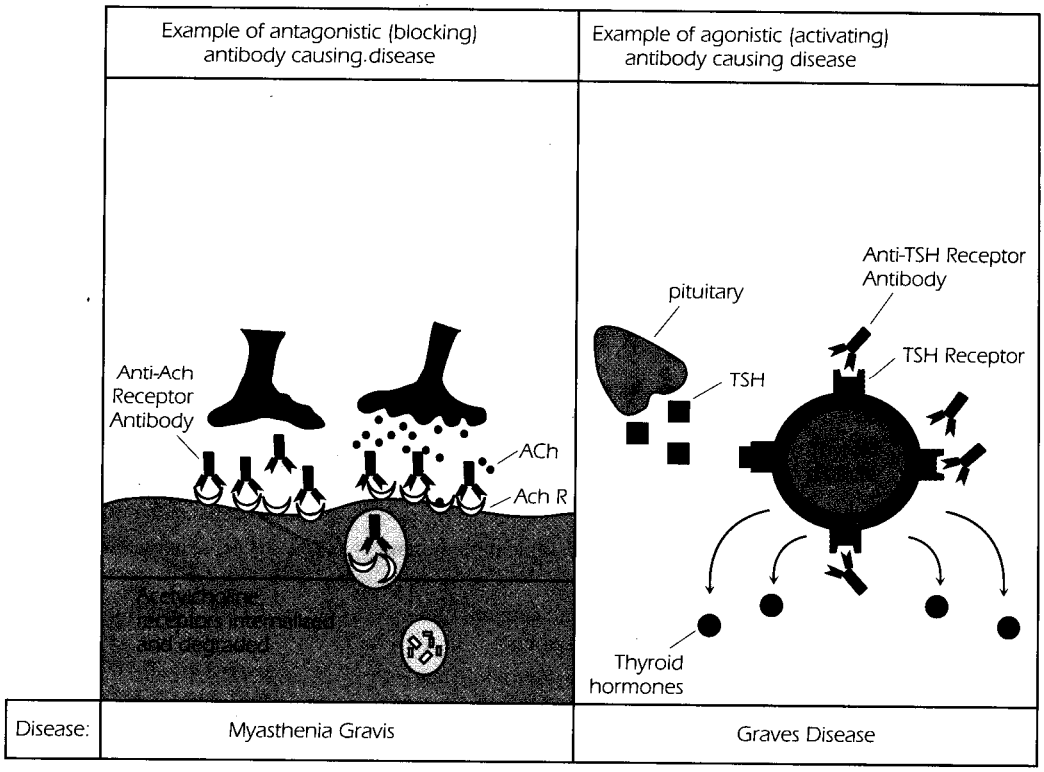

Fig. 8.1. Autoantibodies specific for cell- surface receptors can be either receptor agonists or antagonists. The antibody to the acetylcholine receptor in myasthenia gravis acts as a antagonist that blocks binding of Ach to the receptor and prevents transmission of the nerve impulse across the neuromuscular junction. The antibody to the TSH receptor in Graves disease acts as a receptor agonist and induces chronic stimulation of the thyroid to release thyroid hormones.

8.2.4. Systemic Lupus Erythematosus

SLE gets its name (literally, 'red wolf') from a reddish facial rash on the cheek, which is a frequent early symptom. However, the distribution of the rash resembles the wings of butterfly rather than the face of a wolf. The designation Wolf-like is thus far-fetched, but the term *systemic* is quite appropriate since the disease attacks many organs of the body and causes fever, join pain and damage to central nervous system, heart, and kidneys. The pathophysiology of the kidney lesions, which cause the most mortality from SLE, is the most clearly understood.

Despite the mystery concerning the origin of this disease, details of the immunologic mechanisms responsible for the pathology are partially known. Patients with SLE produce antibody against several nuclear components of the body (antinuclear antibodies-ANA), notably against native ds-DNA. Occasionally, antibodies are also produced against denature, single-stranded DNA and against nuecleohistones; but clinically, the presence of anti-ds DNA correlates best with the pathology of renal involvement in SLE. Antibodies to ss DNA are produced in normal individuals, but they are generally low-affinity IgM antibodies. They can, however, undergo isotype switching and somatic mutation to result in the production of high affinity IgG antibodies to both single- and double- stranded DNA, provided the B cells are given appropriate T cell help.

Double stranded DNA may become trapped in the glomerular basement membrane through electrostatic interactions with a constituent of the membrane such as collagen, fibronectin, or laminin. The bound ds DNA may then trap circulating IgG anti-ds -DNA

antibodies and lead to the formation of immune complexes. These complexes may activate the complement cascade and attract granulocytes. Alternatively, anti- ds- DNA antibodies may cross-react with glomerular antigens. Deposition of IgG antibodies in the kidney of Lupus patients can be demonstrated by immunostaining a tissue section from the kidney with a fluorescently labeled antibody to human IgG. In the kidney, the extent of inflammatory reaction forms the basis of classifying the kidney pathology. The resulting damage to the kidneys (glomerulonephritis) leads to the leakage of protein (proteinuria) and some times hemorrhage (Hematuria), with symptoms waxing and waning as a rate of formation of immune complexes rises and falls. As the condition becomes chronic, the inflammatory $CD4^+$ T_H1 cells enter the site and attract monocytes, which further contribute to the pathologic lesions.

8.2.5. Type 1 Insulin-Dependent Diabetes Mellitus

Insulin dependent diabetes mellitus (IDDM) is a form of diabetes that involves chronic *inflammatory destruction of the insulin-producing β-islet cells of the pancreas*. In IDDM, the major contributors to *β*-cell destructors are *cytotoxic T cells* and cytokines followed by autoantibodies. Genetic factors include several genes in the MHC class II regions, the insulin gene on the chromosome 11, and at least 11 other non-HLA linked diabetes susceptibility genes. Some HLA class II haplotypes predispose for the disease, and others are protective. For example, approximately 50% of IDDM patients are HLA-DR3/DR4 heterozygotes in contrast to 5% of the normal population. On the other hand, individuals with HLA-DQB1*0602 rarely develop the disease.

An experimental animal model, the NOD mouse, shares many key features with the human disease, including the destruction of pancreatic *β*-islet cells by infiltrating lymphocytes, the association with MHC susceptibility genes, and the transmission by T cells. At least 14 genes contribute to diabetes found in NOD mice. There are, however, notable differences between the human disease and the mouse model. These include the predominance of T cells in NOD mice compared to IDDM in humans and a greater bias to incidence of disease in female mice compared to human disease.

8.2.6. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is characterized by chronically inflamed synovium, densely crowded with lymphocytes, which results in the destruction of cartilage and bone. The inflamed synovial membrane, usually one cell thick, becomes so cellular that it mimics lymphoid tissue and forms new blood vessels. The synovium is densely packed with dendritic cells, macrophages, T, B, and NK cells, and clumps of plasma cells; in some cases, the synovium develops secondary follicles. The pathology in its most intense form is probably the consequence of a mixture if immunopathologic mechanisms, specially, antigen-antibody complexes, component, polymorphonuclear neutrophils, inflammatory $CD4^+$ T cells, $CD8^+$ cytotoxic T cells, activated macrophages and NK cells. This "angry mix " releases a variety of cytokines (of which TNF α and IL1 are among the earliest), degradative enzymes, and mediators that destroy the integrity of the cartilage. Chrondocytes the cells of the cartilage, become exposed to the immune system and perpetuate the damage not only by serving as potential targets but also by releasing cytokines and growth factors. Synovial fluid often accumulates in the joints of RA patients and contain large number of polymorphonuclear neutrophils. After repeated bouts of inflammatory insults, *fibrin is deposited*, cartilage is replaced by fibrous tissue, and the joint fuses (*ankylosis*).

It has been suggested that inflammatory processes are initiated by abnormally produced antibody, generally IgM—called *rheumatoid factor* (RF)—*which is specific for a determinant on the Fc portion of the patient's own IgG molecules*. However, it is unlikely that RF is the common initiator of the disease, since 30% of RA patients do not have detectable levels of the factor. The group of patients with RF tends to develop a more aggressive disease. RF serves as a useful marker of disease activity, since reduced levels of serum RF are found during remission. The presence of RF contributes to the pathology of RA but probably does not account for the T cell response.

The initial insulting trigger may be diverse. A high portion of RF patients have elevated number of B cells infected with Epstein-Barr virus; γδ T cells from RA patients recognize heat-shock proteins; and bacteria have been associated with RA. Women are

affected three times more often than men, and the age of onset is usually during the 40s and 50s of age.

8.2.7. Autoimmune diseases of the eye

Phacoanaphylaxis and perforating injuries of the eye are the two autoimmune diseases of eye. Phacoanaphylaxis – is the intraoccular inflammation due to autoimmune response to the lens protein following cataract surgery. Perforating injuries - involving the iris or ciliary bodies are followed by sympathetic ophthalmia in the opposite eye.

8.2.8. Pernicious anaemia

Two types of antibodies are produced, one is directed against parietal cells of gastric mucosa. This is believed to cause achlorhydria and atrophic gastritis. The second antibody is directed against the intrinsic factor and prevent absorption of vit B12. The result is megaloblastic anaemia.

8.3. Control measures of autoimmune diseases

For many years, the major approach to the treatment of most autoimmune disease has been to eliminate autoreactive cells. Because it is not routinely possible to distinguish an autoreactive B or T cell from one that will protect against microbial infection, broadly ablative therapies have been used. Therapeutic agents are often *cytotoxic drugs*, such as *cytoclophosphamide* and *azathioprine*, that interfere with DNA replication and indiscriminately destroy the body's WBC. In addition, drugs like *cyclosporin A* and *FK506* block intracellular signaling pathways and prevent cellular activation.

More recently, *anticytokine therapies* have proven to be very successful in several diseases. Blockade of TNFα by antibody or soluble receptor is an important therapeutic option in rheumatoid arthritis and inflammatory bowel disease. Inhibition of IL-1β by soluble receptor also seems a useful strategy in rheumatoid arthritis. These immunomodulatory agents prevent an inflammatory response. While they appear to

curtail the disease process, they also render the host immunosuppressed. Thus infections represent a major complication of the treatment of many autoimmune diseases. Some autoimmune diseases may be treated by removing or administering a cytokine—for example, interferon-β (INFβ) is used in the treatment of multiple sclerosis. How the cytokine exerts a therapeutic effect is not understood.

Recently, more targeted approaches to therapy have been explored. A nondepleting *monoclonal antibody* to CD3 is being tested in new-onset autoimmune diabetes. Costimulatory blockade, to prevent the interaction of B7 molecules with CD28, appears promising in RA and psoriasis. These new approaches have demonstrated efficacy, but it is likely that they will interfere with protective as well as pathogenic immune responses and thus be immunosuppressive. Additional information about the mechanisms of action of these and other immunosuppressive drugs.

There are some antigen-specific approaches to therapy that may lead eliminate autoreactivity without causing global immunosuppression. Altered peptide ligands, peptides that bind to the MHC groove but are not capable of activating a given T cell, have been used to induce tolerance in rodent models of disease but have not demonstrated efficiency in humans. Oral antigen has also been used to induce tolerance in animal models, but clinical trials with oral collagen and myelin basic protein in RA and MS, respectively, have not demonstrated efficacy. T cell receptors have been administered to patients as an immunogen in an effort to raise clonotype-specific cytolytic T cells. These studies are ongoing.

The recent recognition of multiple populations of regulatory T cells has led to yet another therapeutic strategy. Several studies suggest an absence or a decrease in numbers of T suppressor cells in autoimmune individuals. Investigators are beginning to learn how to generate regulatory cells in autoimmune individuals. There are as yet no clinical trials that are attempting to activate suppressor cells, but in mice this strategy appears quite effective.

8.4. Summary

Autoimmunity is a condition in which the body mounts an immune response to one or more of its own constituents. Establishing a disease as auto immune rests on several types of evidence: 1) direct proof made by transferring autoantibodies or self reactive lymphocytes and reproducing the disease in an otherwise healthy individual; 2) Indirect proof, which requires finding an experimental animal model to mimic the disease; and 3) circumstantial evidence based on familial tendency, involvement of immune cells and antibodies, and clinical improvement with immunosuppressive drugs. Initiation of autoimmune diseases usually requires a combination of genetic and environmental events. It is believed that many autoreactive clones of T and B cells exist normally but are held in check by homeostatic mechanisms. It is the breakdown of these controls, by various mechanisms, that lead to the activation of autoreactive clones and autoimmune disease. Many of organs and tissues are involved in autoimmune disease, and the effecter mechanisms of tissue damage may involve antibody, complement, T-cells, and macrophages.

8.5. Model questions

Write an essay on different types auto immunity diseases.

Write in detail about autoimmunity diseases and their control measures

Write short notes on the following

a) Graves Disease
b) Systemic Lupus Erythematosus
c) Rheumatoid Arthritis
d) Myasthenia Gravis

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Dr. M. RAGHU RAM

Lesson No. 9

BACTERIAL ADHESION

9.0 Objective

The main objective of this topic is to learn and know about the interaction between a bacterial cell and its host cell, different modes of interactive forces act between the cells, different molecules involve in the adhesion process and finally the consequences of the adhesion on both the bacterial and host cells.

9.1 Introduction

A microorganism, in order to remain in or on its host, must first adhere to some cell, secretory product or structural component of its intended host. Hence, bacteria are invariably found on the surfaces of epithelial and epidermal cells, either as a result of direct adhesion to host cells or indirectly by binding to either secretory products which

often coat such cells or to bacteria which have already adhered. Bacteria also adhere to host phagocytic cells and this constitutes one of the host's major defense systems if it results in phagocytosis and destruction of the organism. Once inside the host, a far wider variety of surfaces are available for microbial adherence, including a number of polymers comprising the extracellular matrix, bone, endothelial cells and many specialized cells comprising the various organs of the body.

Collectively, bacteria have evolved an array of surface molecules and structures to enable them to adhere to the cell surfaces. And, a particular organism is usually able to adhere to only a particular surface, or a limited variety of surfaces. Some organisms which are capable of invading tissues have evolved strategies that enable them to adhere to different surfaces as and when required during the course of infectious process. There may be a number of possible outcomes of the bacterium and host interaction. The bacterial adhesion to the host cells may indicate a number of facets including physicochemical forces involvement, specificity of adhesion process, effect on bacteria, effect on host cells and post-adhesion events.

9.2 Basic Principles of Bacterial Adhesion:

9.2.1 Pre-adhesion events:

Several stages are involved in the process of bacterial adhesion to a host cell (Fig. 9.1). At a distance of tens of nanometers, the bacterium and the host cell are influenced by two types of forces namely van der Waals and electrostatic. At a distance of > 50 nm van der Waals interactions occur resulting in the mutual induction of dipoles in two cells which results in mutual attraction between them. When the distance decreases to a range of 10-20 nm, electrostatic forces become significant. As the most bacteria and host cell surfaces have negative charge the net effect is repulsion. But this repulsion force decreases with the increase in ionic strength. And in many natural environments, the ionic strength is sufficient enough to reduce or overcome this repulsion effect. When the bacterium approaches more closely to the host cell, hydrophobic interactions develop between the cells that result in the direct adhesion or bringing the cells close enough (<1.0nm) for the occurrence of other adhesive interactions. These adhesive interactions include hydrogen

bonding, carbon bridging and receptor-ligand interactions. Due to these interactions, a ligand molecule on the bacterial surface specifically binds to a complementary substrate receptor molecule on the host cell surface. Bacterial adhesion to host cells is thought to be mediated primarily by hydrophobic interactions, cation bridging and receptor-ligand binding. The molecules on the bacterial surface responsible for adhesion are known as adhesions.

Fig. 9.1 – Forces affecting bacterial adhesion at varying distances from the host cell surface

9.2.2 Hydrophobic interactions:

When non-polar molecules on the bacterial and host cell surfaces approach one another, the intervening ordered layers of water are displaced and favours the energetic adhesion. In contrast to lectin interactions, hydrophobic bonding is often considered to be non-specific as there are no apparent stereospecific interactions between the molecules. The surface components responsible for the interactions are known as hydrophobins and include hydrocarbon groups, aromatic amino acid groups, fatty acids and mycolic acids. Approximately 55% of the accessible surface of an average protein is non-polar. Even in carbohydrates, which are generally considered to be highly polar, hydrophobic regions do exist. For example, galactose sugars contain six adjacent `C' atoms which form a continuous hydrophobic region and when these sugars interact with proteins, these regions are invariably packed against aromatic side chains in the protein.

9.2.3 Cation-bridging:

The mutual repulsion between the negatively charged surfaces of bacteria and the host cells can be counteracted by divalent metal ions such as calcium ions which thereby act as a bridge between the two cells. Such interactions are thought to be important in adhesion of oral bacteria to tooth surfaces, in co-aggregation between similar and different bacterial species and, possibly between bacteria and negatively charged molecules on the surfaces of host cells.

9.2.4 Receptor ligand binding:

The receptor molecule on the surface of a host cell, whether part of the cell membrane or associated with it, can recognize the ligand molecule on the bacterial surface with a complementary structure and form a strong but non-covalent bond. This process resembles lock-and-key relationship between the catalytic site of an enzyme and its substrate. The recognition process usually involves only a portion of each of the molecules involved and the molecular structure responsible is known as an epitope. A variety of molecules can function as receptors or ligands including proteins, polysaccharides, glycoproteins and glycolipids. When recognition involves the interaction between a protein and a carbohydrate epitope, the interaction is known as lectin binding. The interaction between a receptor and its complementary ligand is highly specific but it can be inhibited by other molecules with identical epitopes.

9.3 Bacterial structures involved in adhesion:

Bacteria elaborate a number of structures which may be involved in adhesion to cell surfaces (9.2). These structures include fimbriae or pili and proteinaceous fibrils whose primary function appears to be that of adhesion, as well as capsules and flagella which have other functional roles, namely protection and locomotion respectively. In addition to these structures, the cell walls of many species contain macromolecules that function as adhesins. A particular species may be able to produce a whole series of adhesive structures or adhesins either concurrently or consecutively. In the latter case, the consecutive production of adhesins may enable the organism to adhere to the different

cell types it encounters during the course of the infectious process in which it participates.

Fig. 9.2 – Bacterial structures involved in adhesion to host cells.

The capsular material is thought to mediate adhesion of a variety of organisms including *Streptococcus* spp., *Staphylococcus* spp., *Klebsiella* spp. and *Bacteriodes* spp. The S layers of bacteria consist of a crystalline array of protein, or glycoprotein, self-assembling units external to the cell wall. This structure is important in adhesion of animal pathogens like *Aeromonas salmonicida* and *Lactobacillus acidophilus* to the host cells than that of human pathogens. The main function of fimbriae is to enable adhesion of the organism to host cells or to other bacteria, the adhesin being located either at the tip of the fimbria or along the length of the fimbrial shaft. Fimbriae are widely distributed among Gram-negative bacterial genera including *Bordetella*, *Salmonella*, *Neisseria*, *Yersinia*, *Pseudomonas*, *Porphyromonas* etc., and have also been detected on streptococci and actinomycetes. Fimbriae constitute the most frequently used adhesive structure in Gram-negative bacteria. As these structures protrude some distance from the cell surface beyond the capsules and other surface molecules, their ability to interact with host cell receptors may help to overcome electrostatic forces of repulsion between the two cells.

Flagella, though are primarily responsible for bacterial motility, also facilitate adhesion in some species like *Pseudomonas aeruginosa*, *Helicobacter pylori* and *Campylobacter jejuni*. The cell walls of bacteria contain a number of molecules which have been shown to have a role in adhesion. The LPS, Outer membrane proteins, Teichoic acids and Lipoteichoic acids all function as adhesins. Besides these, hydrophobic molecules in the

cell wall are also thought to be involved in adhesion by interacting with hydrophobic molecules on host cell surfaces or on the surfaces of inanimate substrata.

9.4 Bacterial adhesins:

A variety of molecules on the surface of the bacterial cell may function as adhesins and mediate attachment of the organism to a host cell or structure. One of the most extensively studied type of bacterial adhesion mechanism involves the interaction between a bacterial lectin and its corresponding receptor on a substratum. Lectins may be found on the end of pili, in capsules or attached to the bacterial cell wall and have been studied mainly in Gram-negative bacteria. Lectin-carbohydrate interactions have been shown to be involved in the adhesion of bacteria to intestinal epithelial cells, pharyngeal epithelial cells, buccal epithelial cells, erythrocytes, urinary tract epithelial cells, other bacteria and teeth.

In a number of Gram-positive bacteria including *Streptococcus pyogenes, Staphylococcus aureus* and *Staphylococcus epidermidis*, the cell wall component namely lipoteichoic acid has been shown to be an important adhesin. In each of these organisms, the receptor for the lipoteichoic acid is fibronectin, a glycoprotein produced by a number of host cells including epithelial cells. Lipoteichoic acids consist of chains of glycerol phosphate, with D-alanine and sugar substituents, attached to a glycolipid or diglyceride moiety which is embedded in the cytoplasmic membrane.

Proteins that are present on the bacterial surface may also function as adhesins. *Staphylococcus aureus* produces a 210 kDa surface protein which mediates adhesion to fibronectin. The same organism also known to bind to a number of other host proteins including fibrinogen, mediated by a 59 kDa protein, and laminin, mediated by a 57 kDa protein. *Streptococcus pneumoniae* adheres to glycoproteins of the nasopharyngeal epithelium mediated by a 37 kDa surface protein called PsaA produced by it. Proteins characterized by proline-rich composition are the major adhesions of *Mycoplasma* species. Recently, an additional heat shock or stress protein namely chaperonin 60 has

been identified on the surface of bacteria including *Helicobacter pylori* and *Haemophilus ducreyi* which involves in the binding of these bacteria to epithelial cells.

Carbohydrates are known to be common components of the bacterial cell surface and may function as adhesions in certain bacteria. An exopolysaccharide of *Pseudomonas aeruginosa* namely alginate appears to play a role in adhesion of the organism to tracheal cells and mucins as it binds to both buccal and tracheal cells and to bronchotracheal mucin. *Streptococcus oralis*, a major constituent of dental plaque, adheres to the tooth surface by means of a polysaccharide composed of hexasaccharide repeating units containing glycerol. A number of surface polysaccharides in *Staphylococcus* epidermidis are known to be the adhesins.

Several studies have implicated Lipopolysaccharide (LPS) as being important in the adhesion of a variety of Gram-negative bacteria. The LPS of *Campylobacter jejuni* is thought to mediate attachment of the organism to epithelial cells. Strains which are highly negatively charged and only weakly hydrophobic bind in greater number to a human intestinal cell line than strains that are more hydrophobic. The binding of *C. jejuni* LPS to the cells can be inhibited by fucose or by treatment of LPS with periodate. LPS is also known to function as an adhesin in other organisms including *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri* and *Escherichia coli*.

There are increasing number of reports of enzymes functioning as bacterial adhesins. The glycolytic enzyme, glyceraldehydes 3-phosphate dehydrogenase, of *Streptococcus pyogenes* is able to bind to a number of proteins on host cells. This enzyme exists as a 156 kDa tetramer on the surface of the organism and mediates binding to fibronectin, lysozyme, myosin and actin. *Porphyromonas gingivalis*, an organism associated with periodontitis, secretes two proteinases, gingipain R and gingipain K which enable the organism to bind to fibrinogen, fibronectin, and laminin. Glucosyltransferase enzymes on the surface of mutans streptococci are thought to be important in the adhesion of these organisms to tooth surfaces. Recently it has also been demonstrated that the enzyme functions in *Streptococcus gordonii* as an adhesin mediating attachment to human

endothelial cells and so may enable colonization of the endocardium in infective endocarditis.

9.5 Nature of the host cell surface:

Broadly considering, bacteria can adhere to the host cell surfaces in three ways – 1) directly to the lipid bilayer, (2) directly to cell surface receptors whose normal function is to bind host molecules, and (3) indirectly to host molecules already bound to the host cell surface. The cytoplasmic membrane of host cells has the classic lipid bilayer structure throughout which proteins are embedded. The major lipids that are present in the cytoplasmic membrane are phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, sphingomyelin, cholesterol and various glycolipids. Several of these contain molecular structures that can be recognized by bacterial adhesins. Although outnumbered by lipid molecules, proteins are responsible for most membrane functions including transport of molecules, recognition and binding of hormones, cytokines and extracellular matrix molecules and signal transduction and cell-cell interactions. Certain amino acid sequences of proteins and the carbohydrates of glycoproteins also function as important receptors for bacterial adhesins.

Mammalian cells display a wide variety of surface receptors for a range of molecules including hormones, immunoglobulins, cytokines and extracellular matrix molecules. Bacteria can adhere to many of these molecules and, as this often induces uptake of the organism by the cell. Host tissues consist of cellular elements embedded in an extracellular matrix (ECM), which comprises a complex mixture of polymers including fibronectin, fibrinogen, collagens, proteoglycans and glycosaminoglycans. Bacteria can also adhere to the components of this ECM of the host cell. Different sites on the host cell surface which can function as receptors for bacterial adhesions are given in figure 9.3 below.

Fig. 9.3 Receptor sites on host cell surface for bacterial adhesins

Some examples of the ECM components of host cell to which bacteria can adhere by their adhesins are given in the table 1.1 given below.

Table 1.1 : Adhesion of bacteria to extracellular matrix molecules

Extracellular Matrix	**Organism**	**Adhesin**
Fibronectin	*Staphylococcus* spp.	110 kDa protein
	Streptococcus pyogenes	120 kDa protein
	Mycobacterium spp.	32 kDa protein
Collagen	*Staphylococcus aureus*	133 kDa protein
	Streptococcus mutans	16 kDa protein
Elastin	*Staphylococcus aureus*	40 kDa protein
Laminin	*Streptococcus gordonii*	142 kDa protein
	Escherichia coli	Carbohydrate
	Helicobacter pylori	25 kDa protein
Fibrinogen	*Staphylococcus aureus*	92 kDa protein
	Streptococcus pyogenes	M protein
	Porphyromonas gingivalis	150 kDa protein

9.6 Effects of adhesion on bacteria:

The adhesion of bacteria to the host cell may bring out some effects on structures as well as functions of bacteria. There are a number of reasons for these effects on bacteria.

Firstly, prior to contacting its host the bacterium is likely to have resided in a number of environments like air, water, soil, vegetation, clothing etc., which may not have been conducive to its growth. On reaching to the epithelial surface the organism will now have to adapt to living, no matter how temporarily, in this new environment and this will involve the upregulation and suppression of a number of gene products. Secondly, attachment to a host cell may be only a preliminary to a whole sequence of events involving invasion of the cell, further dissemination of the organism etc. Attachment of the bacterium might, therefore, be expected to be used as a signal for the organism to commence the synthesis of structures or signaling molecules necessary to elicit invasion of the host cell. Some of the important effects which are given in figure 9.4 include:

1) **Stimulation of bacterial growth** – adhesion to host cells may enhance bacterial growth in some situations. *Neisseria gonorrhoeae*, when attached to HeLa cells grows at a rate three times greater than that of the unattached organism. Similarly, *E. coli* when adherent to epithelial cells derived from the intestine or peritoneal cavity, grows at a faster rate than non-adherent organisms.

2) **Inhibition of bacterial growth** – adhesion of a uropathogenic strain of *E. coli* to human uroepithelial cells has been shown to inhibit further growth of the organism. It was evidentially suggested that calcium and cAMP are involved in the process of bacterial growth inhibition.

3) **Upregulation of siderophore synthesis** – one of the means by which the host restricts bacterial growth *in vivo* is to limit the availability of iron by sequestering it with proteins such as lactoferrin. In order to survive *in vivo*, therefore, bacteria must be able to wrestle iron from host proteins and *E. coli* does this by producing the iron-binding siderophores aerobactin and enterobactin, which are effective at removing iron from host proteins.
4) **Induction of adhesive structures** – adhesion of *Salmonella typhimurium* to an epithelial cells results in the production of surface appendages termed as invasomes by the bacterium. These invasomes are distinctly different from

flagella or pili. The invasomes are thought to be responsible for mediating internalization of the organism by the epithelial cells and disappear once internalization has commenced.

Pathogenic bacteria which attach and infect mucosal surfaces often form discrete micro-colonies which is particularly evident in the case of enteropathogenic strains of *E. coli.* This phenomenon, known as localized adherence is shown to be associated with the induction by the epithelial cells of bundles of filaments on the bacteria.

5) **Secretion of proteins required for invasion** – in the case of *Shigella flexneri*, three proteins namely IpaB, IpaC, and IpaD that involve in invasion of epithelial cells are produced as a consequence of adhesion. Similarly, in *Campylobacter jejuni* as many as 14 proteins are shown to be synthesized for invasion purpose on stimulation by adhesion.

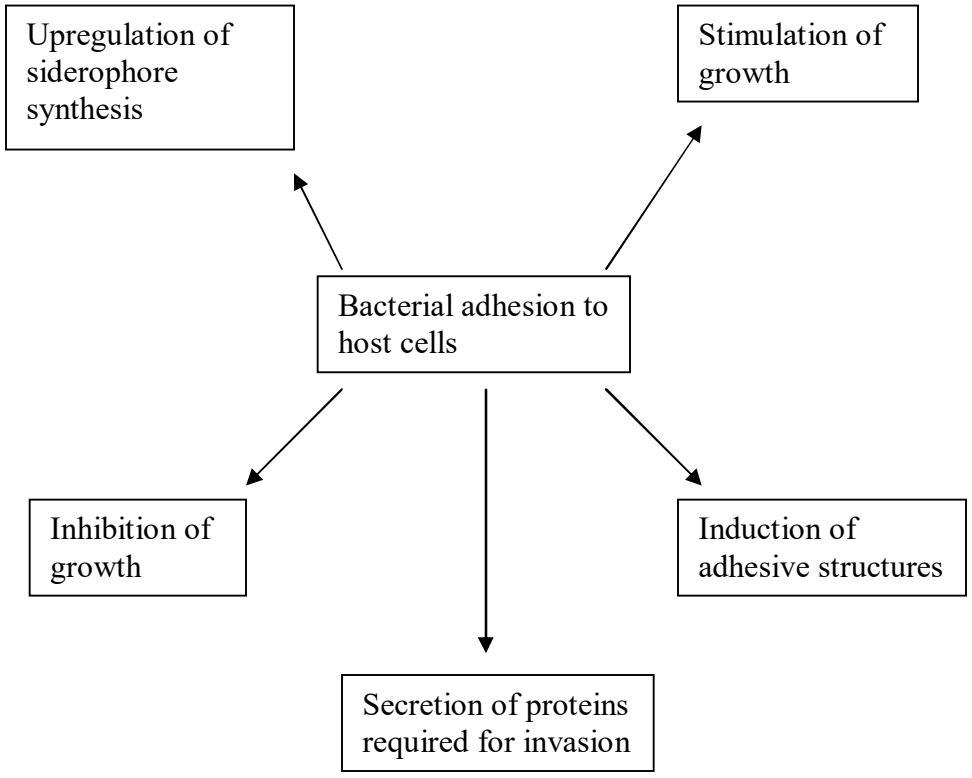

Fig. 9.4- Effects of bacterial adhesion to host cells on bacterial structure / function

9.7 Effects of adhesion on host cells:

The effects of adhesion of bacteria on host cells range from alterations in cell morphology to long-term transcriptional changes to programmed cell death and include an altered morphology, no apparent biological effect, invasion, induction of fluid loss, induction of cytokine release, upregulation of intercellular adhesion molecules and apoptosis (Fig. 9.5). The outcome of the interaction is very much depends on the type of the host cell with which the bacterium interacts. Apart from the alveoli, bladder, stomach and the urethra, all of the epithelial surfaces of a healthy individual are normally colonized by bacteria. In general, most of these adherent organisms, as contain molecules which are potentially harmful to mammalian cells, have minimal effects on epithelial cells.

Colonization without any apparent biological effect – It is generally assumed that colonization of the mucosal surfaces of the intestinal epithelium by *Bacteriodes* spp., enterococci and *Eubacterium* spp. and of the vagina by lactobacilli does not induce any dramatic changes in the behaviour of either member of the association.

Induction of morphological alterations – Enteropathogenic *E. coli* (EPEC) strains are the major cause of diarrhoea in children worldwide. The organism does not produce any enterotoxins or cytotoxins and is not usually invasive but induces a characteristic attaching and effacing (A/E) lesion in epithelial cells in which microvilli are lost and the underlying cell membrane is raised to form a pedestal which can extend outwards for up to 10 μm.

Induction of cytokine release – A wide variety of streptococcal species are the numerically dominant genera of bacteria in the oral cavity and most of them rarely cause disease. Adhesion of any of 11 strains of oral streptococci to epithelial cells was found to stimulate release of the CXC chemokine and interleukin 8. The binding of *E. coli* to urinary epithelial cells via P,S or type 1 fimbriae has been shown to elicit the release of the proinflammatory cytokines Il-6, IL-8, IL-1α and IL-1β.

Expression of intercellular adhesion molecules – adhesion of *E. coli* to epithelial cells induces the release of the PMN (Polymorphonuclear leukocyte) chemoattractant IL-8, which induces the migration of PMNs from the bloodstream. At the same time, the adherent *E. coli* upregulates the expression of ICAM (Intercellular adhesion molecule) on the surface of the cells which binds to the CD11b/CD18 integrin on the surface of the arriving PMNs.

Cellular invasion – Adhesion of pathogenic bacteria to epithelial cells often is a preliminary to invasion. The adhesion of bacteria itself often triggers a series of changes in the host cell leading to uptake of the organism.

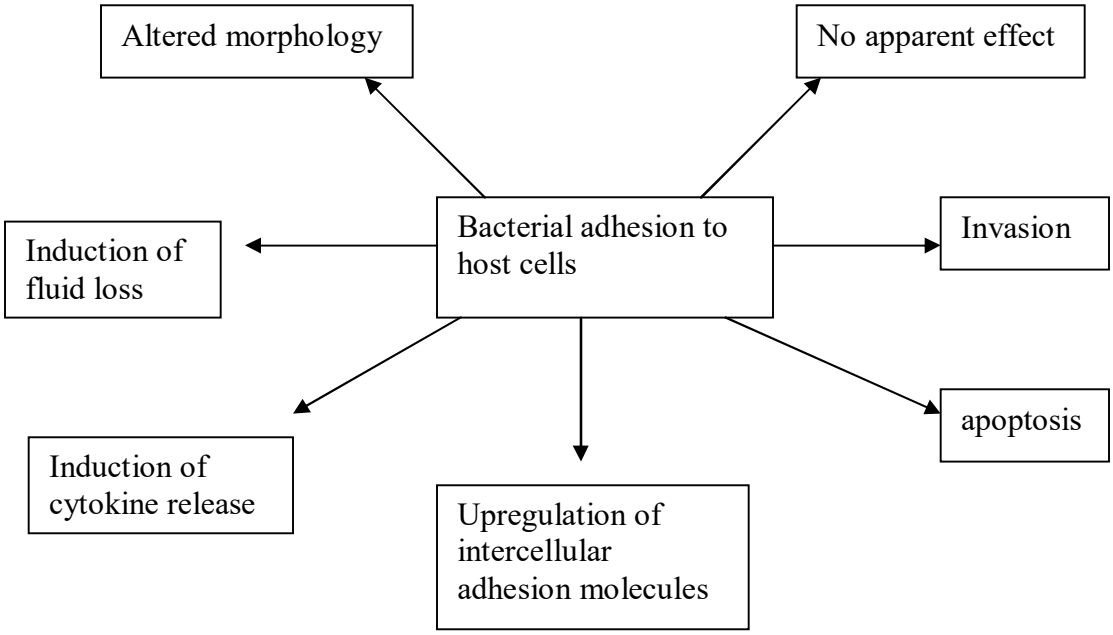

Fig. 9.5 - Effects on host cell structure / function of bacterial adhesion

9.8 Summary:

The first stage in any infectious process is adhesion of the causative organism to a host cell or to polymers secreted by host cells. Bacteria synthesize adhesins which enable them to bind to specific receptors on the target cell or secreted polymers. Once

bound to their target cell or molecule, bacteria engage in an exchange of signals with their host which can have one of several consequences viz., (i) mutual indifference and the continued existence of both members of the association in an, apparently, unaltered state, (ii) profound alterations to the target cell resulting in either localized or systemic pathology, (iii) invasion of the host cell and induction of pathology elsewhere.

9.9 Model questions:

1. Define adhesion? Explain the basic principles and molecular mechanisms of bacterial adhesion.
2. Describe the consequences of the bacterial adhesion to host cell on both the partners.
3. Write a note on bacterial adhesions.

9.10 Reference books:

1. Cossart, P., Boquet, P., Normark,S. and Rappuoli, R (2000) – Cellular Microbiology – ASM Press, Washington, USA

2. Henderson, B., Wilson, M., McNab, R. and Lax, A J (1999) – Cellular Microbiology—John Wiley & Sons, New York, USA

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Lesson No. 10

BACTERIAL INVASION

10.0 Objective

The main aim of this lesson plan is to understand the routes and mechanisms of bacterial invasion of host cells.

10.1 Introduction

The general dictionary definition of the word *invade* is `to enter for hostile purposes'. The presence of a bacterium inside a host cell may evidence the occurrence of invasive process. In the case of non-phagocytic cells such as epithelial cells, the presence of bacterium within them is a clear cut evidence for invasion. But the situation may not be a

clear cut in the case of phagocytic cells such as macrophages. Hence, the presence of bacteria within a macrophage may indicate that the host cell is merely performing its normal function, i.e., disposing of bacteria, or it may indicate that the macrophage is being invaded, as for some bacterial species this is their preferred habitat.

Invasion of host tissues by bacteria is a characteristic feature of a number of infectious diseases. The first stage in an invasive process is the adhesion of the invading organism to a host cell. The invasion enables bacteria to evade host defenses, seek out new supplies of nutrients or find new tissues to colonize. Bacteria, collectively, have evolved a variety of invasive mechanisms, some common elements in these processes are evident as most involve the manipulation of normal host cell cytoskeletal components such as actin and tubulin, resulting in the invagination of the host cell membrane to enclose the bacterium within a vacuole. This often occurs by interference with the intracellular signaling pathways either by stimulation or inhibition of signal transduction, or both.

Among the invasive pathogens, more diversity is evident when considered the events subsequent to invasion. Hence, the organism may remain inside the vacuole or escape from the vacuole and colonize the cytoplasm or escape from the vacuole and the cell and then spread systemically. The adoption of an intracellular life-style confers several advantages to microbial pathogens; they become inaccessible to humoral and complement-mediated attack, they have no longer require a specific adherence mechanism to maintain their site of infection, and they have ready access to a range of nutrients. However, aspiring intracellular pathogens must develop specific strategies to secure and maintain they life-style. The majority of mechanisms exhibited by intracellular pathogens are the product of evolutionary selection in response to their intracellular existence.

10.2 Routes of Invasion

To infect a cell, a microbe must first adhere to it. For some pathogens, this adherence phase determines the choice of the cell to be infected, while for other, although they are capable of binding to many cell types, development of the pathogen is restricted to only

certain cell lineages. Adherence and host cell entry are tightly associated phenomena, and so considered together as functions of the route and mechanism of invasion. There are three basic mechanisms of invasion : (i) Phagocytosis, i.e., entry into professional phagocytes such as macrophages, monocytes, and neutrophils via a process dependent on the host cell contractile system; (ii) Induced endocytosis and phagocytosis, i.e., entry into non-professional phagocytes by the active induction of internalization through the activity of the host cell contractile system; and (iii) Active invasion, i.e., active entry into a passive host cell without triggering any contractile event in the host cell cytoskeleton (Fig. 10.1).

Fig. 10.1 – Different routes of invasion

10.2.1 Phagocytosis

Many microbial pathogens are capable of either transient or sustained infection of professional phagocytes. Macrophages and other phagocytes represent the frontline defense of the host against microbial invasion. These cells migrate through tissues, internalizing and degrading foreign particles and this behavior will obviously maximize the frequency of interaction between phagocytes and microbes. The macrophage has receptors which recognize a range of ligands including the serum opsonins antibody and complement. Pathogens that activate the alternate pathway of complement will accumulate C3b and iC3b on their surface. The macrophage is equipped with high-affinity receptors viz., CR1 and CR3/CR4, respectively, for these ligands on the pathogen. So, these opsonized bacteria will bind to and be internalized by the phagocyte. The only specific mechanism required by the pathogen is to facilitate complement

deposition while avoiding lysis by insertion of the terminal membrane attack components. Pathogens such as *Salmonella* and *Leishmania* achieve this through elongation of their surface lipopolysaccharide or lipidoglycans, respectively. These long carbohydrate chains activate complement but avoid lysis because the activating convertase is maintained some distance from the outer membrane. Complement receptors trigger phagocytosis without stimulating a strong superoxide burst from the macrophage. In phagocytosis mode of invasion, the infecting pathogen is relatively passive in the process following ligation to host cell receptors capable of triggering internalization. This process requires little if any metabolic activity from the parasite. Examples include *Leishmania*, *Mycobacterium* and *Histoplasma.*

10.2.2 Induced Endocytosis and Phagocytosis

In induced endocytosis and phagocytosis, the pathogen induces a normally nonphagocytic cell to internalize the microbe. The pathogens that infect nonprofessional phagocytes have evolved their own ligands for adherence to host cells. Host cell entry by many of these pathogens is a facilitated process that involves the induction of an internalization response in the by the adherent microbe. This is the least well understood route of entry and involves subversion of the host cell signaling pathways. One of the best studied example of these pathogens is *Salmonella*, which induces the actin cytoskeleton to from extensive membrane ruffles, or splash, during its entry into the cells. This response is accompanied by the phosphorylation of several host cell proteins as a consequence of activation of host cell signaling cascades.

10.2.3 Active invasion

In contrast to the pahagocytosis and induced endocytosis mechanisms, active invasion involves invasion of the hast cell without triggering any contractile events such as phagocytosis. In active invasion, the pathogen invades the host cell without the participation of the contractile apparatus of the host cell. In this process, the host cell is inert. Examples include all the apicomplexan parasites, *Plasmodium, Toxoplasma*, *Eimeria* and microsporidia. In fact, for *Toxoplasma*, the ability to block the maturation of the entry vacuole into an acidic, lysosomal compartment is determined at the time of host

cell entry. All apicomplexan parasites, including *Plasmodium, Toxoplasma*, and *Eimeria*, have motile invasive stages called zoites. These stages show an actin-based motile system and that mediates both gliding mobility and host cell invasion.

Recent work with mutant host cells and Toxoplasma lines resistant to the antimicrofilament agent cytochalasin D has shown that mutant parasite lines invaded host cells in the presence of the drug irrespective of the phenotype of the host cell. If this invasion process was subverted by opsonizing the parasites with immunoglobulin G (IgG), the Fc receptors on the host cell which were ligated during invasion prevented the parasites from maintaining their vacuoles outside the endosomal continuum. The vacuoles acidified, and the parasites died. Conversely, if *Toxoplasma* was allowed to invade and infect host cells and was subsequently killed by treatment with pyrimethamine, an inhibitor of the parasite's dihydrofolate reductase activity, the vacuoles persisted as isolated intracellular compartments, presumably because the host cell membrane fusion apparatus could not recognize them. Because *Toxoplasma* is promiscuous in its ability to invade a range of different host cells, the ligand on the host cell must be virtually ubiquitous. Although no ligand has been identified formally, experiments have implicated extracellular matrix proteins.

10.3 Mechanisms of bacterial invasion

The human body is not a single organism but, rater, a complex ecological community, most of whose members are bacteria. When all the bacteria found on the skin, in the nose and mouth, in the lower gastrointestinal tract, etc., are taken into account, there are about 10 times more bacterial cells than human cells in the body. However, the immune system keeps them corralled in appropriate locations, and most internal tissues are normally sterile. Opportunistic pathogens must await an injury or other breach in the barriers before they can reach these niches., but some primary pathogens have developed mechanisms to sneak across them. With respect to manipulation of the host cell cytoskeleton, several interesting examples are found among pathogens that invade epithelial cells.

Two epithelia – the lining of the intestines and the lungs – are particularly vulnerable to attack by bacterial pathogens. Since the function of these epithelial is to absorb digested nutrients and oxygen, respectively, and pass them into the bloodstream, the barrier is a cellular monolayer. Bacteria that can invade this monolayer are one step away from access to the bloodstream and the rest of the body. Epithelial cells take up proteins and other factors from the outside, using pinocytosis and receptor-mediated endocytosis, but they are generally not capable of engulfing large objects such as bacteria. To be taken up by the epithelial cells, the bacteria must induce their own phagocytosis.

There are two recognized mechanisms by which pathogenic bacteria induce phagocytosis by epithelial and other nonphagocytic cells; these are termed the Zipper and Trigger mechanisms (10.2). Both of these mechanisms are actin dependent and require that the extracellular bacteria induce intracellular polymerization and reorganization of actin filaments at the plasma membrane. In both cases, the bacteria manipulate the host cell signal transduction apparatus to induce cytoskeletal responses similar to those that occur in response to natural stimuli. The zipper mechanism exploits pathways normally involved in cellular adhesion, while the trigger mechanism resembles the membrane ruffling response to eukaryotic cell growth factors.

Fig. 10.2 – Zipper and Trigger mechanisms

10.3.1 Zipper mechanism of bacterial uptake by Non-phagocytic cells

10.3.1.1 *Listeria monocytogenes* – One bacterial pathogen that is a recognized champion at manipulating the host cell actin cytoskeleton is *Listeria monocytogenes*. *L.*

monocytogenes is a gram-positive food-borne pathogen that can cause serious infections in pregnant women and immunocompromised people, resulting in a relatively high rate of mortality among infected adults, newborns, and fetuses. In healthy adults, the infection can be asymptomatic or result in mild, flu-like symptoms. *Listeria* is usually found in soil, in water, and on plants but can be isolated from animals as well, and infections can be transmitted in foods such as unpasteurized dairy products. In addition, *Listeria* can contaminate stored refrigerated foods due to its unusual ability to grow at 4^{o}C.

Once ingested, some *Listeria* organisms pass through the upper gastro-intestinal tract to the small intestine, where they are thought to enter the intestinal epithelium. In tissue culture models of infection, *Listeria* can invade a wide variety of cell types, including epithelial, endothelial, and fibroblastic cells, by using a zipper mechanism. This term refers to the ability of the bacterium to induce very local changes in actin organization which result in a close apposition of the bacterium to the host plasma membrane. The bacterium appears to sink into the cell, or to pull the plasma membrane up around it, in a process that does not involve formation of membrane ruffles as in the trigger internalization process and apparently does not require more than a very local rearrangement of the actin cytoskeleton.

Two bacterial factors called internalins have been identified that allow *Listeria* to zipper into eukaryotic cells. Internalin A (InlA) is an 88-kDa bacterial surface protein that contains leucine-rich repeats that are thought to participate in protein-protein interactions. InlA is necessary for invasion of cultured intestinal epithelial cells, and transposon insertion into the inlA gene abolishes the invasive ability of *Listeria*. During invasion of epithelial cells, InlA interacts with a host receptor, the E-cadherin molecule. E-cadherin is an adhesion protein responsible for homotypic association of epithelial cells. It is a transmembrane protein whose cytoplasmic side is linked to the actin cytoskeleton through a complex of proteins which includes α-, β-, and γ-catenin. It is not known whether these interactions are important for *Listeria* invasion, but they could represent a link between InlA and the cytoskeletal changes that results in *Listeria* internalization.

In other cell types, InlA is not required for *Listeria* invasion; it has been shown that the 65-kDa protein InlB is responsible for the internalization of bacteria into cultured hepatocytes, Chinese hamster Ovary (CHO) cells, and HeLa cells. The host cell receptopr for InlB has not been identified. *Listeria* harbors at least four other genes containing leucine-rich repeats that may function as internalins for other host cell types. Perhaps the diversity of the internalin multigene family reflects the diversity of their potential cellular targets; cadherins, too, are a diverse multigene family.

Internalization of *Listeria* by the zipper mechanism involves a dynamic actin cytoskeleton in the host cell as well as the activation of tyrosine kinases and lipid kinases. Inhibitors of tyrosine kinases and the lipid kinase phosphatidylinositol 3-kinase (PI3-K) block entry of *Listeria* into epithelial cells in culture. Cytochalasin D, an inhibitor of actin polymerization, also blocks bacterial entry. Interestingly, cytochalasin D does not block the activation of PI3-K, suggesting that PI3-K may act upstream of the signals to the cytoskeleton and may be involved in the activation of that signal. The precise steps of PI3-K activation and those leading to actin rearrangement in *Listeria* invasion have to yet been uncovered. Different steps involved in invasion are given in the figure 10.3 below.

Fig. 10.3 Stages involved in the invasion of epithelial cells by *L. monocytogenes*

10.3.1.2 *Yersinia* species – an analogous zipper entry mechanism may be used by some species of the gram-negative pathogen *Yersinia*. *Y.enterocolitica*, *Y.pseudotuberculosis*,

and *Y.pestis* are the causative agents of mild to severe bouts of gastroenteritis, animal disease similar to that seen in human patients, and bubonic plague, respectively. *Y.enterocolitica* and *Y.pseudotuberculosis* enter the body by the fecal-oral route and invade the mesenteric lymph nodes around the intestines through the Peyer's patches. In tissue culture models of infection, these species are capable of invading epithelial cells. A *Yersinia* outer membrane protein, invasin, binds tightly to the subset of integrins that harbor a β_1 subunit. Integrins, like cadherins, are adhesion proteins, but they generally mediate adhesive interactions between cells and the extracellular matrix. As with *Listeria*, inhibitors of tyrosine kinases and actin polymerization inhibit *Yersinia* invasion.

Both these examples of zipper-type mechanism invasion demonstrate bacterial exploitation of mechanisms normally involved in host cell adhesion. Cadherins and integrins mediate attachment of epithelial cells to and spreading on their neighbors and the underlying extracellular matrix, respectively. Engagement of these receptors by their normal ligands results in receptor immobilization and clustering. These events induce signaling cascades that can result in strengthening of the cell-to-cell and cell-to-matrix contacts and in cellular differentiation. When a bacterial surface protein engages the adhesion proteins on a host cell, the host cell responds as it normally would, recruiting cytoskeletal elements to the location of the attachment and attempting to strengthen the attachment. However, since the bacterium is small compared to the responding cell, the attempt of the cell to spread against the bacterial surface quickly results in engulfment of the bacterium. This striking example of functional convergence suggests that it may be relatively easy for a bacterial pathogen to develop the ability to invade epithelial cells by a zipper mechanism. The only requirement appears to be that the bacterium express a surface protein that binds to a cell surface adhesion receptor with an appropriate affinity. The steps in the invasion process are given in figure 10.4 below.

Fig. 10.4 Stages involved in the invasion of epithelial cells by *Yersinia* spp.

10.3.2 Trigger Mechanism of Bacterial uptake by Non-phagocytic cells

10.3.2.1 *Shigella* species – The second mechanism of bacterially induced phagocytosis, the trigger mechanism, is dramatically different from the zipper mechanism in morphological terms and requires a much more complicated type of bacterial machinery. In these cases, brief contact between a bacterium and the surface of a host cell results in a rapid, large-scale cytoskeletal response, where explosive actin filament polymerization under the plasma membrane pushes out huge sheets or ruffles. The extending ruffles fold over and fuse back to the cell surface, trapping large membrane-bound pockets of extra-cellular medium within the cell, a process termed macropinocytosis. The nearby bacteria are trapped by the infolding membrane ruffles, and the bacteria then find themselves within the membrane-bound compartments. Where zippering invasion appears to be a modification of cellular adhesion, triggering invasion bears much more resemblance to the response of cells to growth factors.

Shigella is a gram-negative pathogen that is the causative agent of bacillary dysentery. There are four species of *Shigella* that cause dysentery:*S. dysenteriae*, *S. sonnei*, *S. boydii*

and *S. flexneri*. They have similar mechanisms of virulence, and since *S. flexneri* is the best studied, the molecular descriptions presented here are for *S.flexneri* is the best studied, the molecular descriptions presented here are for *S. flexneri* but presumably also apply to the other species. *Shigella*-mediated dysentery is usually a self limiting although unpleasant diarrheal disease in healthy adults, but kills about 500,000 children every year in developing countries, where contaminated water is all that is available. *Shigella* infection can occur at a very low dose of bacteria, with a 50% infectious dose of 100 to 200 organisms, probably because the bacteria are not killed by stomach acid and find their way unharmed to the colon. During *Shigella* infection, the bacteria can invade a variety of cell types including macrophages, M cells, and the intestinal epithelia, and after escaping from the phagocytic vacuole, they can move within and between cells by actin-based motility.

The trigger mechanism of *Shigella* invasion involves proteins whose genes are carried on a 220-kb virulence plasmid. Bacteria that have been cured of this plasmid lose invasive ability. The main system involved in this process is a type III secretion system that is activated when the bacteria come into contact with the host cells. This is not a process of transcriptional activation but, rather, mobilization of proteins already existing in the cytosol of the bacterial cell. The genes involved in this process are called the ipa (invasion plasmid antigens) and the Mxi-spa (membrane expression of ipa-surface presentation of antigens) genes. The Mxi-spa genes encode the secretory apparatus, while the ipa genes encode the secreted proteins. The protein products, IpaB, IpaC, and IpaD, are all necessary for *Shigella* invasion. IpaB and IpaC are secreted into the extracellular milieu and are part of a complex that is sufficient for invsion of the host cells. This ‘Ipa complex’ does not contain IpaD but may contain other factors.

The host cell factors involved in *Shigella* invasion are those that are normally involved in the formation of membrane ruffles in response to growth factors and other signals that cause morphologically similar actin-rich structures to form. One study has suggested that the $\alpha_5\beta_1$-integrin may be the host cell receptor for the Ipa complex, but this has not been definitively proven. It remains possible that the Ipa complex acts by creating a pore

through which it inserts effector proteins or that some other interaction with the cell membrane is necessary. The ruffles that form upon *Shigella* invasion extend actin-rich membrane projections near the site of the bacterial contact that contain the actin-bundling protein fimbrin.

The regulatory signals responsible for the actin rearrangements that occur in *Shigella* invasion include activation of the low-molecular- weight GTPase Rho. Members of the Rho family of small GTPases mediate actin cytoskeletal reorganizations in response to extracellular growth factors and act as molecular switches in a variety of signal transduction pathways. In firbroblasts, Rho induces the formation of stress fibers and focal adhesions in response to growth factors. Another signaling molecule that may be involved in this process is the tyrosine kinase $pp60^{c\text{-}src}$. Upon invasion by *Shigella,* c-Src phosphorylates one of its major substrates, cortactin, and cortactin is recruited to the actin-rich projections that are induced. Cortactin is an actin-binding protein of the cortical actin cyrtoskeleton and may be the connection between the activation of the c-Src tyrosine kinase and the actin cytoskeleton. The steps in the invasion process are given in figure 10.5 below.

Fig. 10.5 Stages involved in the invasion of epithelial cells by *Shigella* spp.

10.3.2.2 *Salmonella* species – *Salmonella* species are responsible for food-borne general gastroenteritis (*S. typhimurium*) as well as more specific ailments such as typhoid fever (*S. typhi*). *Salmonella* bacteria are gram-negative pathogens closely related to *Escherichia coli.* One of the important aspects of the virulence of *Salmonella* is its ability to pass through the intestinal epithelia and invade underlying cells including enterocytes and macrophages. Interaction of *Salmonella* with host cells causes the generation of membrane ruffles and macropinocytosis, resulting in trigger-type internalization. Invasion of epithelial cells allows *Salmonella* to colonize and cross the barrier monolayer in the intestine. In more serious infections, *Salmonella* also invades and grows inside the macrophages. Apparently, induction of triggered uptake by the macrophages preempts their normal phagocytic activity. Whereas *Salmonella* could not survive in a ormal macrophage phagosome, since the phagosome is quickly targeted to fuse with lysosomal degradative compartments, the spacious membrane-bound compartment that *Salmonella* generates for itself by triggering ruffling and macropinocytosis can support bacterial replication.

A number of host cell signaling pathways that may be responsible for the rearrangement of the actin cytoskeleton that occurs upon interaction of *Salmonella* with the host cell membrane have been identified. In Henle 407 cells (a human epithelial tissue culture line), interactions of *Salmonella* with the cells in culture cause the activation of the epidermal growth factor receptor (EGFR) tyrosine kinase. Activation of this receptor triggers the mitogen-activated protein kinase, and this results in activation of phospholipase A_2 (PLA_2). Lipid products of PLA_2, such as arachidonic acid, can have effects on actin regulatory proteins, perhaps by interacting with the regulators of the Rho subfamily of GTP-binding proteins. The eicosenoid leukotriene D_4 (LTD_4), which is generated from arachidonic acid by PLA_2, may also play a role in ruffle formation by *Salmonella*. Generation of LTD_4 results in a rise in the intracellular calcium concentration that is common to many pathways that activate changes in the actin cytoskeleton. Several actin-severing proteins are regulated by calcium and their activation make them for the rapid actin polymerization at the plasma membrane that

accompanies *Salmonella* invasion. The steps in the invasion process are given in figure 10.6 below.

Fig. 10.6 Stages involved in the invasion of epithelial cells by *Salmonella* spp.

In some cell types, such as HeLa and B82 fibroblast cells, other pathways have been identified as important for *Salmonella* invasion. The B82 fibroblasts have no EGFRs on their cell surface. In these cell types, activation of PLC-γ results in the formation of inositol-1,4,5-triphosphate (IP_3) and release of calcium form intracellular stores. This is distinct from the situation in Henle cells, which activates the influx of extracellular calcium. The bacterial proteins that are responsible for the activation of these host cell pathways have not been definitively identified, but some clues have been provided by the demonstration that *Salmonella* has a type III secretion system similar to that identified in *Yersinia* and *Shigella* species. A number of gene products that are indispensable for *Salmonella* invasion have been identified. Some of these. Like InvJ and SpaO, appear to be a part of the secretory apparatus, while others are likely to be the effectors responsible for the rapid cytoskeletal changes that occur when *Salmonella* interacts with the host cell. One bacterial effector protein secreted upon *Salmonella* invasion that has been studied is the SptP tyrosine phosphatase. SptP acts with other secreted effectors to elicit

the disruption and reorganization of the cytoskeleton that occurs upon *Salmonella* invasion.

Despite the morphological and mechanistic differences between the trigger and zipper mechanisms for bacterial invasion of non-phagocytic cells, they have certain important traits in common. They include

- Both require that extracellular bacteria send signals across the host cell plasma membrane to induce local actin polymerization.
- Both involve bacterial activation of signal transduction cascades that are already present in the host cell although used for other purposes.
- Both are mediated by bacterial expression of specific virulence factors, whose sole function is to induce these particular responses during host cell invasion.
- Importantly, both zipper and trigger uptake proceed by using energy derived from the host cell.
- The cytoskeletal and membrane rearrangements that are responsible for bacterial invasion require no energetic input from the bacterium; they invade by persuation rather than by force.

10.4 Consequences of bacterial invasion on host cell

Invasion of a host cell by a bacterium can affect the former in a variety of ways, ranging from the barely perceptible to the ultimate effect i.e., death. In may cases, however, the effects are intermediate between these extremes and may be transient or long-lived depending mainly on whether the bacterium takes up permanent residence within the cell. The consequences include cytokine release, prostaglandin release, cell death etc.

Cytokine release – A variety of cells respond to bacterial invasion by secreting cytokines and the release of these inflammatory mediators may serve to activate host defence systems or, because of their overproduction, may have adverse consequences for the host.

The best example is the secretion of four proinflammatory cytokines namely IL-8, monocyte chemoattractant protein (MCP)-1, GM-CSF and TNFα in response to invasion by *Salmonella dublin*, *Shigella dysenteriae*, *Yersinia enterocolitica*, *Listeria monocytogenes* and enteroinvasive *Escherichia coli*.

Prostaglandin release – Infection of intestinal epithelial cells with the invasive organisms *Salmonella dublin*, *Shigella dysenteriae*, *Yersinia enterocolitica* or enteroinvasive *Escherichia coli* both in vitro and in vivo has been shown to rapidly upregulate the secretion of prostaglandin.

Cell death – Intracellular growth and replication within host cells often lead to the death of the colonized cell as a result of nutrient depletion, degradation of important intercellular constituents and the build-up of toxic end products of bacterial metabolism which is often termed necrotic cell death. However, bacterial invasion of host cells can sometimes induce a second form of cell death known as apoptosis which is also called as programmed cell death.

10.5 Summary

Human physiology has evolved physical barriers and complex and specific immune responses to kill the pathogenic bacteria with which we come into contract. Bacteria have evolved to circumvent these defenses in a variety of different ways, using our own biochemistry and cellular makeup to their advantage to find niches through which to attack us. One of the host cell systems that has been exploited by a number of different bacterial species is the actin cytoskeleton. The actin cytoskeleton is a meshwork of protein filaments that extend throughout the cytosol and form a dense web underlying the plasma membrane. In most vertebrate cells, the actin cytoskeleton is the primary determinant of cell shape and provides machinery for whole-cell movement. The actin cytoskeleton is involved in many signaling mechanisms in response to both intracellular and extracellular signals and can reorganize quickly to change local morphology or global cell shape.

The importance of the actin cytoskeleton to eukaryotic cells and its responsivenss to a wide variety of signals have provided pathogenic bacteria with a plethora of chances for opportunistic exploitation. There are different ways that different pathogens attack the eukaryotic cells, invade into and manipulate the cytoskeletal systems of the host cells. Phagocytosis, induced endocytosis and active invasion are the important routes of invasion that a pathogen may access into the host cell. The non-phagocytic cells take up the bacterial pathogens by two mechanisms namely Zipper mechanism and Trigger mechanism. The two mechanisms are having differences on one side and on the other side they exhibit some common or similar features.

10.6 Model Questions

1. What is invasion? Explain the different routes of bacterial invasion.
2. Describe the molecular mechanisms of bacterial invasion with suitable examples.
3. Short answers:
   (i) Zipper mechanism
   (ii) Trigger mechanism
   (iii) Consequences of bacterial invasion on host cell

10.7 Reference Books

1. Cossart, P., Boquet, P., Normark,S. and Rappuoli, R (2000) – Cellular Microbiology – ASM Press, Washington, USA

2. Henderson, B., Wilson, M., McNab, R. and Lax, A J (1999) – Cellular Microbiology— John Wiley & Sons, New York, USA

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Lesson No. 11

SECRETION SYSTEMS

11.0 Objective

The main aim of this lesson plan is to know about the types of secretion systems in bacteria, type III secretion apparatus and type IV secretion system mediated T-complex transfer system in *A. tumefaciens*.

11.1 Introduction

A multitude of activities performed by bacteria, as well as eukaryotic cells, are dependent upon the transport of proteins through membranes. In bacteria, these activities include the synthesis of extra cellular organelles such as flagella, the degradation of nutrients too large to ingest directly, and the delivery of toxins directed against eukaryotic cells to their site of action. Transporting a protein through a lipid bilayer is not a trivial task, considering the relative physical dimensions of proteins compared to small solutes such as water and sodium ions. This task is further complicated by the fact that most proteins destined to be transported across a membrane must be prevented from assuming their three-dimensional structure prior to transport. Pathogenic bacteria of humans and plants have coopted conjugation systems to export virulence factors to eukaryotic host cells. Although this is a functionally diverse family, there are some unifying themes: (i) exporters are assembled at least in part from subunits of DNA conjugation systems, and

(ii) the known substrates recognized by these transporters are large macromolecules such as nucleoprotein particles and multi-subunit toxins.

11.2 Types of Secretion Systems

Gram-negative bacteria possess several protein secretion systems (Fig. 11.1). The best studied systems at the mechanistic level are the sec-dependent type II and V secretion systems, which most probably represent evolutionarily ancient systems since homologues of the components of these secretion machines also exist in gram-positive bacteria, the archaebacteria, and the eukaryotes. Proteins secreted by these systems possess N-terminal signal sequences of 16 to 26 residues that consist of a basic N-terminal domain, a hydrophobic central core segment, and a distal domain that contains a cleavage site in which the signal sequence is removed during the transport across the inner membrane. The basic and hydrophobic domains are essential for the recognition event between the protein to be transported and components of the secretion complex located on the cytoplasmic face of the inner membrane. Following this initial recognition event, the protein destined to be secreted is transported across the inner membrane by a mechanism that has been extensively studied and is fairly well understood. After transport across the inner membrane, a protein may either remain in the periplasm, integrate in the outer membrane, or continue its journey across the outer membrane. Proteins secreted by the type II and V pathways differ from each other in how they traverse the outer membrane. Proteins secreted by the type V secretion system, which include the immunoglobulin A proteases of *Neisseria gonorrhoeae* and the *Helicobacter pylori* vaculating cytotoxin, mediate their own secretion across the outer membrane. In contrast, proteins secreted by the type II secretion system, such as the extracellular degradative enzyme pullulanase of *Klebsiella oxytoca*, require additional proteins for their secretion across the outer membrane.

Proteins secreted by the type I secretion system are mainly toxins such as hemolysin of *Escherichia coli* and cyclolysin produced by *Bordetella pertussis*. Unlike proteins secreted by the type II secretion system, these proteins contain no signal sequence at their N termini but instead contain domains at their C termini that are necessary for recognition

by the type I secretion complex. This complex is relatively simple, consisting of only three proteins: an ATPase that provides the energy for transport as well as forms a pore through the inner membrane, a protein that spans the periplasmic space, and a protein located in the outer membrane. Both the nature of the type I secretion signal and the transport mechanism are currently unclear.

Type IV secretion systems in gram-negative bacteria are specialized for the cell-to-cell transfer of a variety of biomolecules. This secretion system is used for the transfer of protein-DNA complexes between either two bacterial cells (during bacterial conjugation) or between a bacterial cell and eukaryotic cell (*Agrobacterium tumefaciens*-mediated DNA transfer into plant cells). Additionally, *B. pertussis* and *H. pylori* possess type IV secretion systems which mediate the secretion of pertussis toxin and interleukin-8-inducing factor, respectively. The structural basis of the type IV secretion machine that allows it to secrete polynucleotide as well as polypeptide molecules is not known.

Several gram-negative bacteria that interact sith either plant or animal cells possess a secretion system, referred to as the type III secretion system, that functions to deliver proteins directly from the bacterial cell into the cytosol of the eukaryotic cell. This secretion system was first described and characterized in Yersinia spp. that are pathogenic for humans. Genetic analysis of the 70.5 kb virulence plasmid of Yersinia species revealed that it encoded both the proteins that were secreted and approximately 20 proteins that comprised the secretion complex.

Type III secretion systems have been found in several genera of bacterial animal and plant pathogens. Interestingly, it has recently become apparent that nonpathogenic plant-infecting bacteria utilize type III secretion systems as well. The nitrogen-fixing plant symbiont *Rhizobium* uses a type III secretion system to secrete proteins involved in the formation of rood nodules. Remarkably, it appears that in a few cases there are similarities between the proteins secreted by the type III secretion systems of plant- and animal-infecting bacteria. Among the bacterial species possessing type III secretion systems, there is a high degree of similarity between the various type III secretion

systems both in terms of genetic organization and at the level of the individual genes. The genes encoding the type III secretion apparatus are clustered in blocks that largely display a conserved genetic order. Among the individual genes themselves, which encode the type III secretion apparatus, some are found in virtually all species while others are more restricted in their distribution. Thus, type III secretion systems from different species can be grouped into subfamilies based on sequence similarities and genetic organization.

Divergence of type III gene sequences between species could be due, at least in part, to different selection pressures that are imposed on the bacteria during their coevolution with their respective hosts. In this scenario, a particular bacterial species would first acquire a type III-like secretion system and then modify that system, through natural selection, to fit its specific needs. The intestinal pathogen *Salmonella* provides an illustrative example of the adaptation of type III gene clusters. This pathogen possesses two entirely separate type III secretion systems, encoded by different gene clusters, which, based on genetic evidence, apparently were acquired at different times during evolution. These two systems are required for different stages of the *Salmonella* infection process. One system secrets proteins involved in the invasion of eukaryotic cells, while the other system is required for the bacterium to survive once it is inside the eukaryotic cell. It is currently unclear why *Salmonella* requires two independent type III secretion pathways.

Fig. 11.1 – Different types of bacterial secretion systems

11.3 Secretion Apparatus

Flagella are extracellular appendages that mediate bacterial motility. A flagellum is composed of three structural components : a thin helical filament of variable length, a hook, and a basal body. The basal body is embedded in the bacterial envelope and serves to anchor the flagellum filament to the bacterial surface via the hook protein linkage. The basal body consists of a central continuous rod surrounded by several ring structures. The L ring resides in the outer membrane, and the P ring is embedded in the peptidoglycan layer. The MS ring is situated in the inner membrane, and the bell-shaped C ring, harboring the motor/switch proteins (FliG, FliM, and FliN), is associated with the MS ring in the inner membrane but projects into the cytoplasm. Another component of the flagellar biosynthetic machinery consists of a family of proteins located in either the inner membrane or the cytoplasm, which collectively function as the flagellum-specific secretion apparatus responsible for the sequential secretion of proteins forming the rod, hook, and filaments.

Interestingly, structures that contained proteins known to be part of the SPI-1 (for *Salmonella* pathogenicity island 1) encoded type III secretion complex of *Salmonella* were isolated and revealed features resembling the flagellar basal body. These structures were observed only on bacteria deficient in flagellum production but expressing a functional type III secretion system. In particular, the part embedded in the bacterial envelope appeared physically similar to the ring structures in the inner and outer membranes of the basal body. The proteins constituting the MS and C rings in the basal body have been identified, but these display only limited similarity to particular proteins of the type III secretion systems of pathogenic bacteria. Nevertheless, in view of the basal body-like structures of *Salmonella*, it is likely that proteins specific to type III secretion systems form similar ring structures in the bacterial inner membrane.

A significant feature of the *Salmonella* type III secretion complex is that instead of the extracellular flagellar appendage, a hollow needle-like structure, 80 nm long and 13 nm wide, extends from the bacterial surface. This needle-like structure may function to bridge the gap between the bacterium and the host cell to establish translocation of

virulence proteins across the host cell plasma membrane. In support of this, a 7–to 9-nm-diameter, EspA-containing filamentous surface organelle of enteropathogenic *Escherichia coli* (EPEC) physically connects bacteria to infected eukaryotic cells. This structure, encoded by the EPEC type III secretion system located on the pathogenicity island designated LEE (for "locus of enterocyte effacement"), is required for translocation of effector proteins into the target cell. By analogy, there is a substantial amount of indirect evidence suggesting that proteins secreted by the type III secretion machinery of plant-interacting bacteria, like bacterium-animal cell systems, are injected directly into the plant cell cytoplasm.

While there is no significant similarity among proteins comprising the flagellar basal body and type III secretion systems, seven proteins that constitute the flagellum-specific secretion apparatus show extensive similarity to particular proteins of type III secretion systems. These flagellum-specific secretion proteins localize in, or are associated with, the inner membrane and appear to be linked to the basal-body ring structures, possibly localizing to the central pore inside the MS ring. And it is very likely that a similar structure is formed by protein components of the type III apparatus specific for secretion of virulence proteins. Additionally, based on the observation of continuous secretion by the type III complexes, it has been postulated that a flagellar rod-like structure is associated with the ring structures in the inner membrane and extends through the periplasm to the outer membrane. However, no proteins with significant sequence similarity to the flagellar rod proteins FlgB, FlgC, FlgF and FlgG have been observed for any component of type III secretion systems. Some of the proteins are found unique to type III secretion systems involved in forming a structure that connects the postulated rings in the inner and outer membranes. The comparative structures of flagellum and type III secretion apparatus are given in the below figure 11.2.

Fig. 11.2 – Structures of flagellum and *Salmonella* type III secretion apparatus

Although most proteins of type III secretion systems appear to be located in the bacterial inner membrane or cytoplasm, a subfamily of proteins including YscC and MxiD, from *Yersinia* and *Shigella*, respectively, are clearly located in the outer membrane and are required for type III secretion. The emerging picture for the mechanism of type III secretion is that proteins destined for secretion are identified and targeted to the secretion apparatus at the inner membrane via a mechanism similar to that for flagellar proteins. The additional proteins of the secretion apparatus may be required to gate pores present in both the inner and outer membranes connected by a channel extending through the periplasm, as well as to provide the energy required to channel the proteins to the exterior.

11.4 *A. tumefaciens* T-Complex Transfer System

The best-characterized type IV system is that used by *Agrobacterium tumefaciens* for exporting oncogenic DNA (T-DNA) to plant cells. The result of DNA transfer is the proliferation of plant cells and ultimately the formation of tumorous tissues termed crown galls. T-DNA transfer requires approximately 20 virulence (Vir) proteins, which are encoded by six operons. Some of the Vir proteins, notably the VirD2 endonuclease and the VirE2 single-stranded-DNA-binding protein (SSB), interact with single stranded T-DNA (the T strand) to form the transfer intermediate (the T complex). Other Vir proteins,

including the 11 virB gene products and the virD4 gene product, assemble into a gated channel-pilus complex. The channel serves as a transenvelope conduit through which the T complex passes, while the pilus is proposed to mediate the physical contact between *A. tumefaciens* and recipient plant cells. The VirB components of the T-DNA transport system are highly related to Tra protein components of transfer systems encoded by several broad-host-range plasmids.

Extensive studies of the *A. tumefaciens* T-complex transporter components have generated the following information. VirB1, a probable trans-glycosylase, is a nonessential virulence factor, but a truncated, exported form of the protein (VirB1*) has been postulated to play a role in mediating specific contacts with recipient cells. There is compelling evidence that VirB2 is the major pilin subunit for the pilus (T pilus). VirB2 localizes at the inner membrane, and, upon an unknown signal, a processes form is recruited to the outer membrane for pilus polymerization. The VirB proteins are required for pilus assembly, but it is not known whether these proteins participate directly in pilus morphogenesis or simply provide an anchor at the membrane for pilus attachment. VirB4 and VirB11 contain consensus Walker "A" nucleotide-binding motifs, and each hydrolysis ATP in vitro. These proteins utilize the energy of ATP hydrolysis to drive transporter assembly or substrate translocation. Each of these ATPases forms a homodimer early during assembly with other transporter components, although the final oligomeric structure of these proteins in the fully assembled transporter is unknown. Both ATPases are tightly associated with the inner membrane. VirB4 is an integral membrane protein with two domains either embedded into or through the membrane into the periplasm. VirB11 is tightly bound to the membrane and most probably is localized exclusively on the cytoplasmic face of the membrane. The functions of the remaining transporter components have not been defined. Several integral inner membrane protein components such as VirB6 and VirB10 are likely candidates as structural components of an inner membrane pore. Other outer membrane components such as VirB7 and VirB9 are likely candidates as components of an outer membrane pore. The remaining VirB proteins may be structural subunits of the putative transenvelope channel, or they may transiently assist in the assembly of this structure.

The possible roles of individual VirB proteins in transporter assembly have been analyzed in part by construction of non-polar virB mutants. Studies of these mutants revealed that some of the VirB proteins, most notably VirB6, VirB7, and VirB9, provide important stabilizing functions for other VirB proteins. VirB7, an outer membrane lipoprotein, was shown to stabilize itself as well as VirB9 through the assembly of a disulfide cross-linked VirB7-VirB9 heterodimer. Several lines of evidence suggest that the correct positioning of the VirB7-VirB9 heterodimer at the outer membrane is required for assembly of a functional transporter. Thus, it has been proposed that this heterodimer functions as a nucleation center for the transporter assembly. Of further interest, VirB6, a polytopic inner membrane protein, recently has been shown to facilitate VirB7 dimer formation or stabilization, as well as the stabilization of other VirB proteins including the VirB4 and VirB11 ATPases. Interestingly, the PtlI and PtlF homologous of VirB7 and VirB9 also assemble as disulfide cross-linked dimers, and dimmer formation also is important for Ptl protein stabilization. Furthermore, the PtlD homolog of VirB6 has a stabilizing effect on other Ptl transporter components. Finally, the PtlH homolog of VirB11 requires an intact ATP-binding domain for function, supporting the sequence-based prediction that PtlH provides energy from ATP hydrolysis for transporter assembly or function.

11.5 Summary

One very sophisticated approach that gram-negative bacteria have evolved to manipulate eukaryotic cells involves direct injection of proteins into the cytosol of the infected cell. These bacterial proteins injected by the type III secretion system either impede or activate particular eukaryotic cellular responses depending on what is beneficial for the bacterium. The variety of species that possess type III secretion systems, ranging from animal pathogens to plant symbionts, suggest that animal- and plant-interacting bacteria have common strategies to overcome, or at least survive, the eukaryotic defense system. Knowledge of the type III secretion system and the proteins it injects will undoubtedly allow the design of novel therapeutic strategies for use against microbial pathogens. For example, the Salmonella type III system has been used to inject viral proteins into eukaryotic cells that are eventually displayed on the eukaryotic cell surface in association

with MHC proteins. The resulting surface presentation of MHC-viral peptides promotes the development of the adaptive immune response that serves to protect the animal from subsequent viral challenge.

The evolution of a family of secretion systems from ancestral DNA conjugation machines raises many interesting questions and exciting new research directions. Equally important are detailed mechanistic and comparative studies of the type IV transporters with respect to the definition of (i) the basis for substrate recognition, (ii) the architectural arrangement and assembly pathway(s), (iii) the energy requirements for transporter assembly and function, and (iv) molecular interactions between these systems and eukaryotic host cells. Such information ultimately will be useful for applied studies aimed, for example, at designing drugs, for selective inactivation of type IV secretion systems, or, even more enticing, at testing the potential of these systems for delivery of therapeutic protein or DNA macromolecules directly to eukaryotic cells.

11.6 Model Questions

1. Explain the different types of secretion systems in bacteria and secretion apparatus of Type III secretion system.
2. Describe the *A. tumefaciens* T-complex transfer through type IV secretion system.

11.7 Reference Books

1. Cossart, P., Boquet, P., Normark,S. and Rappuoli, R (2000) – Cellular Microbiology – ASM Press, Washington, USA

2. Henderson, B., Wilson, M., McNab, R. and Lax, A J (1999) – Cellular Microbiology— John Wiley & Sons, New York, USA

Lesson No. 12

BACTERIAL TOXINS

12.0 Objective

The aim of this lesson plan is to understand the categorization and mode of action of some important types of bacterial toxins.

12.1 Introduction

Toxins were the first bacterial virulence factors to be identified and were also the first link between bacteria and cell biology. Over 300 toxins have been characterized, produced by many different genera of bacteria, and they are found both in Gram-positive and Gram-negative bacteria. Cellular microbiology was naturally born a long time ago

with the study of toxins and expanded to include the study of many other aspects of bacterium-cell interactions. In the instances, where the bacteria free supernatants fully reproduced the symptoms of deadly diseases such as diphtheria, tetanus, cholera and botulinum, the bacterial toxins were the only factors needed by bacteria to cause disease, making the study of their pathogenesis straightforward. In many cases, immunity against the toxins is enough to prevent the disease caused by the bacterium that produces them, and therefore they have been often used as vaccines after being subjected to chemical treatment to remove their toxicity.

The powerful effects of toxins, such as cell death, and the striking morphology changes that they caused in vivo in animal models and in vitro on cells made it easy to study their interactions with eukaryotic cells. For this reason, toxins were the first link between bacteria and cell biology. The link became stronger when it was found that the discovery of any toxin target led to the discovery of a new, important pathway in cell biology. This is because toxins need targets consisting of molecules that pay a key role in the most essential and vital processes of living organisms. Two very important pathways of cell biology were discovered due to bacterial toxins and they have been extensively investigated. The first is the G-protein mediated signal transduction, which was discovered in the early 1980s and it was understood only because of pertussis toxin that specifically blocked the inhibitory G proteins. The second pathway is the molecular mechanism of neurotransmitter release, which was discovered when tetanus toxin was found to specifically cleave vesicle-associated membrane protein, a key molecule in intracellular vesicular trafficking.

Toxins have a target in most compartments of eukaryotic cells. For simplicity, the toxins can be divided into three main categories namely –

- Those that exert their powerful toxicity by acting on the surface of eukaryotic cells simply by touching important receptors, by cleaving surface-exposed molecules, or by punching holes in the cell membrane thus breaking the cell permeability barrier
- Those that have an intracellular target and hence need to cross the cell membrane

- Those that are directly delivered by the bacteria into eukaryotic cells.

12.2 Toxins Acting on the Cell Surface

Some toxins deliver their toxic message just by binding to receptors on the surface of eukaryotic cells. The most common of these toxins are a family of related molecules known as superantigens. The binding domains of (B domains) of the AB toxins sometimes also belong to this group. Some toxins act by cleaving important molecules exposed on the surface of eukaryotic cells. Another group of molecules that bind to the cell surface and in many cases affect cell function contains the pore-forming, which act by punching holes in the cell membrane and breaking the cell permeability barrier.

12.2.1 Superantigens

Superantigens are produced mostly by *Staphylococcus aureus* and *Streptococcus pyogenes.* They are bivalent molecules that bind two distinct molecules, the major histocompatibility complex (MHC) class II molecules and the variable part of the T-cell receptor (Fig. 12.1). The cross linking between the MHC and the T-cell receptor is able to activate T cells even in the absence of a specific peptide. Therefore, superantigens are potent polyclonal activators of T cells both in vitro and in vivo. In vivo, the potent polyclonal activation results in a massive release of cytokines such as interleukin-1 and tumor necrosis factor, which are believed to play an important role in diseases such as the toxic shock syndrome induced by toxic shock syndrome toxin 1 (TSST-1), vomiting and diarrhea caused by staphylococcal enterotoxins, and the examthemas caused by the pyrogenic streptococcal exotoxins.

The staphylococcal enterotoxins (SE) comprise a family of homologous proteins of approximately 230 amino acids, with one central disulfide bond, named staphylococcal enterotoxins A through H (SEA, SEB, SEC1, SEC2, SEC3, SED, SEF, SEG, and SEH). TSST-1 also belongs to the same family but is shorter (194 amino acids) and has no cysteines. The streptococcal erythrogenic toxins include streptococcal pyrogenic enterotoxin A and C (SPEA and SPEC) and streptococcal superantigen A (SSA).

Other bacterial toxins have been proposed to be superantigens. One of them is the streptococcal pyrogenic exotoxin B (SpeB), a virulence factor with cysteine protease activity produced by group A streptococci. SpeB is expressed as a 40-kDa protein and is converted to a 28-kDa active protease by proteolytic cleavage. It causes a cytopathic effect on human endothelial cells and represents a critical virulence factor in human infection and in mouse models of invasive disease.

A role proposed for the cysteine protease activity of SpeB is a posttranslational modification of the *Streptococcus* surface-expressed M protein. Removal of 24 amino acids from the N terminus of mature M protein alters the bacterial immunoglobulin-binding properties. Sequence analysis of the SpeB gene in 200 group A *Streptococcus* isolates has identified three main variants.

Fig. 12.1 Superantigen interaction with MHC class II molecule and T-cell receptor

12.2.2 Pore-Forming Toxins

Pore-forming toxins work by punching boles in the plasma membrane of eukaryotic cells, thus breaking the permeability barrier that keeps macro molecules and small solutes selectively within the cell. These toxins are often identified as lytic factors (lysins). Since erythrocytes have often been used to test the activity of these toxins, many of them are known as hemolysins. However, it could be kept in mind that hemolytic acitivity was only a convenient way to measure their ability to punch holes in the cell membrane and

release the intracellular hemoglobin, whose red colour is easy to detect even with naked eye. Therefore, while erythrocytes are a convenient target of hemolysins in vitro, they are never the main physiological target of this class of toxins in vivo, since the toxins exert their virulence effect mostly by permeabilizing other cell types. To generate channels and holes in the cell membrane, this class of proteins must be amphipathic, with one part interacting with the hydrophilic cavity filled with water and the other interacting with the lipid chains of the non-polar segments of integral membrane proteins. The ultimate consequence of cell permeabilization is usually death, whereas the early consequences of losing the permeability barrier are often the release of cytokines, activation of intracellular proteases, and sometimes induction of apoptosis. The oligomerization of large and small pore forming toxins have been given in figure 12.2 below.

Fig. 12.2- Oligomerization of large-pore forming toxins (A) and small-pore forming toxins (B)

12.2.2.1 Large Pore-Forming Toxins

Streptolysin O (SLO) is the best known member of a toxin family containing 19 different secreted proteins of 50 to 60 kDa that have the common property of binding specifically to cholesterol on the cell membrane. They contain a common motif located approximately 40 amino acids from the carboxy terminus. Oxidation of the cysteine residue, included in this motif, abolishes the lytic activity; this activity can be restored by

adding reducing agents such as thiols. These proteins are produced by *Streptococcus pyogenes*, *S. pneumoniae*, *Bacillus*, a variety of clostridia including *Clostridium tetani*, and *Listeria* (Table 12.1). They all bind cholesterol-containing membranes in a non-saturable fashion and then polymerize to form very large pores, which can be up to 35 nm in diameter and which make the cell membrane permeable to small solutes and largne macromolecules, thus leading to rapid cell death. Resolution of the three-dimensional structure of perfringolysin O, a member of this family produced by *C. perfringens*, has revealed a novel mechanism of membrane insertion and pore formation. The membrane-bound receptor, cholesterol, plays a significant role in monomer targeting, oligomerization, membrane insertion, and stabilization of the membrane pore. SLO is widely used as a tool in cell biology to produce in vitro large pores in cell membranes, thus allowing the introduction of large molecules into the cell cytoplasm.

Bacterial genus	**Species**	**Name of the toxin**
Streptococcus	*S. pyogenes*	Streptolysin O
	S. pneumoniae	Pneumolysin
Bacillus	*B. cereus*	Cereolysin O
	B. alvei	Alveolysin
	B. thuringiensis	Thuringiolysin O
Clostridium	*C. tetani*	Tetanolysin
	C. botulinum	Botulinolysin
	C. perfringens	Perfringolysin O
	C. septicum	Septicolysin O
	C. bifermentans	Bifermentolysin
	C. histolyticum	Histolyticolysin O
Listeria	*L. monocytogenes*	Listeriolysin O
	L. ivanovii	Ivanolysin

Table 12.1 Large Pore-forming Toxins

12.2.2.2 Small Pore-Forming Toxins

The small-pore-forming family of toxins form very small pores (1 to 1.5 nm in diameter) in the membrane, allowing the selective permeabilization of cells to solutes that have a molecular mass lower than 2,000 Da. Alpha-toxin and leukotoxins belong to a family of related 33-kDa toxins secreted by most pathogenic strains of *S. aureus*. This family

contains leukotoxins such as leukocidin F, leukocidin S, and α-lysin A and C, which bind to the membranes, where they assemble into heptameric structures that form very small pores (approximately 1 nm in diameter). The X-ray structure of the transmembrane pore of alpha-toxin has been solved and has confirmed the heptameric structure of the oligomer and the self assembly of the glycine-rich region to form the pore. Cells become permeable only to molecules smaller than the pores (up to 2,000 Da). Ions like Ca^{2+} are able to enter the cells, so that electrical permeability is established, while large molecules such as all cytoplasmic enzymes are retained within the cell. At this stage, the toxin induces a number of changes in the cell such as production of eicosanoids, activation of endonucleases, release of cytokines, and early apoptotic events. At high concentrations, the toxin can cause membrane rupture and cell lysis, thus killing the cells. The toxin causes membrane damage in a variety of cells including erythrocytes, platelets, and leukocytes. In erythrocytes, for instance, the intoxication proceeds in distinct steps: binding to cell membrane, ion leakage, and eventually rupture of the cell membrane with release of larger molecules.

Aerolysin is secreted by *Aeromonas hydrophila* as a 52-kDa protoxin that is activated by proteolytic cleavage of the carboxy-terminal peptide to yield a 48-kDa active toxin. The toxin binds to a family of GPI-anchored specific receptor on the surface of target cells, including the T-cell receptor RT6. Following binding, the protein oligomerizes and forms heptameric strucrtures that insert into the cell membrane, forming pores of approximately 1.5 nm in diameter.

12.3 Soluble Toxins with an Intracellular Target

The group of toxins with an intracellular target (A/B toxins) contains many toxins with different structures that have only one general feature in common i.e. they are composed of two domains generally identified as A and B. A is the active portion of the toxin; it usually has enzymatic activity and can recognize and modify a target molecule within the cytosol of eukaryotic cells. B is usually the carrier for the A subunit; it binds the receptor on the cell surface and facilitates the translocation of A across the cytoplasmic membrane. Depending on their target, these toxins can be divided into different groups

that act on protein synthesis, signal transduction, actin polymerization, and vesicle trafficking within eukaryotic cells.

12.3.1 Toxins Acting on Protein Synthesis

The toxins that inhibit protein synthesis, causing rapid cell death, at extremely low concentrations are diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, and Shiga toxin.

12.3.1.1 Diphtheria toxin

Diphtheria toxin (DT) is a 535 amino acid polypeptide encoded by a bacteriophage that lysogenizes *Corynebacterium diphtheriae*. The gene is regulated by an iron-binding protein, and therefore the toxin is expressed only in the absence of iron. Following cleavage at a protease-sensitive site, the toxin is divided into two fragments (A and B) that are held together by a disulfide bridge. The A fragment (DTA) is an enzyme with ADP-ribosylating activity that binds NAD and transfers the ADP-ribose group to elongation factor 2 (EF2) according to the reaction shown below:

$$\text{NAD} + \text{EF2} \xrightarrow{\text{DTA}} \text{ADPR-EF2} + \text{nicotinamide} + \text{H}^+$$

The target site in EF2 is a unique amino acid resulting from the posttranslational modification of histidine at position 715; it is named diphthamide. The region containing diphthamide 715 is very close to the anticodon recognition domain of EF2. This suggests that ADP-ribosylation interferes with EF2 binding to the tRNA. The ADP-ribosylated EF2 is no longer able to support protein synthesis, and the cells die. The lethal dose of DT is extremely low: 100 ng/kg of body weight. The B fragment can be further divided into two domains : the R (receptor binding) and T (transmembrane) domains. R binds to the heparin-binding epidermal growth factor-like precursor and is internalized by receptor-mediated endocytosis. The toxin receptor is present in most mammalian cells; however, rodents are not susceptible to DT because the receptor has a few amino acid changes that abolish binding. Following acidification of the endosomes, the T domain, which is composed mostly of hydrophobic α-helices, changes conformation and penetrates the membrane and somehow facilitates the translocation of the A subunit to

the cytoplasm. DT is used to make the diphtheria vaccine, where it is present after chemical detoxification by formaldehyde treatment.

Another activity described for DT is apoptosis of target cells. This activity is apparently mediated by the A fragment but is not linked to the enzymatic activity. In fact, apoptosis has also been described for cross reacting material (CRM197), an enzymatically inactive, nontoxic mutant of DT. Whether apoptosis plays a role in toxicity in vivo is unclear; however, it cannot be a major role because the mutants active in apoptosis but enzymatically inactive are nontoxic in vitro and in vivo.

12.3.1.2 *Pseudomonas* Exotoxin A

Pseudomonas exotoxin A (ExoA) is a 66-kDa single chain protein with a mechanism of action (ADP-ribosylation of EF2) identical to that of Diphtheria Toxin. However, the two proteins are totally unrelated and have no primary sequence homology. Nevertheless, the folding and three-dimensional structure of the catalytic site are conserved and superimposable on those of all mono-ADP-ribosylating enzymes known, including the A fragments of Diphtheria Toxin, Cholera Toxin, Labile Toxin, and Pertussis Toxin, suggesting that these enzymes either evolved from a common ancestor or had a convergent evolution.

The toxin is secreted into the supernatant by *P. aeruginosa* and can be divided into three functionally different domains: the amino-terminal part, R, binds the α_2-microglobulin receptor on the surface of target cells; the central portion, T, is composed mostly of hydrophobic α-helix and mediates translocation of the catalytic domain to the cytosol; and the carboxy terminal part has ADP-ribosylating activity. Following receptor-mediated endocytosis, the toxin undergoes retrograde transport to the endoplasmic reticulum, where it crosses the membrane and is translocated to the cytoplasm to reach the EF2 target.

12.3.1.3 Shiga Toxin

Shiga toxin, also known as verotoxin, is a prototype of a number of related toxins produced by the causative agents of dysentery (*Shigella dysenteriae*) and hemorrhagic colitis (*E. coli* producing Shiga toxins types 1 and 2). The toxins are encoded by an operon which carries the genes for the A and B subunits and can be located in the chromosome or on bacteriophages. Shiga toxin is a typical A/B toxin, with an enzymatically active A peptide of 35 kDa that is responsible for the toxicity. This has an N-glycosidase activity, which cleaves an adenine residue from the 28S RNA, altering the function of the ribosomes, which are no longer able to interact with elongation factors EF1 and EF2. This results in an inability to carry out protein synthesis and cell death. Interestingly, the plant toxin ricin has an identical mode of action. The B subunit is a pentamer composed of five identical monomers of 7,700 Da that bind to the globotriaosylceramide (Gb) eukaryotic cell receptor. The organization of the B pentamer is remarkably similar to that found in CT and LT, suggesting that this type of pentameric structure is favourable and has been independently and recurrently adopted during evolution. After binding to Gb_3, the toxin is internalized by receptor-mediated endocytosis and is transported to the Golgi and to the endoplasmic retuculum, from which the A subunit is translocated to the cytoplasm, where it can gain access to the ribosomal target.

12.4 Toxins Directly Delivered by Bacteria into the Cytoplasm of Eukaryotic Cells

These toxins are the molecules that have been discovered relatively recently and are part of a fascinating, rapidly expanding field. The bacterium that directly deliver the molecule engages in an individual fight with one eukaryotic cell. The final result is not different from what happens after the bite of a poisonous insect or snake or after the release of a potent bacterial toxin into the infected host. These bacteria intoxicate individual eukaryotic cells by using a contact-dependent secretion system to inject or deliver toxic proteins into the cytoplasm of eukaryotic cells. This is done by using specialized secretion systems that in gram-negative bacteria are called type III of type IV, depending on whether they use a transmembrane structure similar to flagella or conjugative pili, respectively.

12.4.1 EPEC Tir

A unique protein acting on signal transduction in eukaryotic cells is Tir (translocated intimin receptor) of enteropathogenic *E.coli* (EPEC). This is a protein containing two predicted transmembrane domains and six tyrosines. The 78-kDa protein is transferred, by a type III secretion system dependent mechanism, to eukaryotic cells, where it becomes an integral part of the eukaryotic cell membrane and functions as receptor for the bacterial adhesin, intimin. At this stage, the protein mediates attachment of bacteria to the eukaryotic cells and is tyrosine phosphorylated, resulting in an apparent molecular mass of 90 kDa. Following tyrosine phosphorylation, the protein mediates actin nucleation, resulting in pedestal formation and triggering tyrosine phosphorylation of additional host proteins, including phospholipase C-γ. Tir is essential for EPEC virulence and is the first bacterial protein described that is tyrosine phosphorylated by host cells.

12.4.2 *C. botulinum* Exoenzyme 3

Exoenzyme C3 is, not a true toxin, a protein of 211 amino acids that is produced by *Clostridium botulinum* and that in vitro ADP-ribosylates the small regulatory protein Rho at asparagine 41, inactivating its function. The enzymatic activity is identical to that of all ADP-ribosylating enzymes. If the protein is microinjected into cells or if the cells are transfected with the C3 gene under a eukaryotic promoter, actin stress fibers are disrupted, the cells are rounded, and arborescent protrusions form. However, it was not clearly known whether C3 alone is able to enter cells and intoxicate them, because no mechanism of cell entry has been found.

12.5 Summary

Bacterial protein toxins are responsible for the damage caused by many pathogens in disease. Toxin expression is often tightly regulated and are then transported from the bacterial cell, often using a complex mixture of proteins to achieve export. Certain toxins act on the cell surface, while others enter the cell, usually in a membrane-bound vesicle. Intracellularly acting toxins are often constructed to exploit the severe conditions there in order to expose previously buried residues which can penetrate into the membrane, and also to effect cleavage to release the fragment with the catalytic toxic activity. The active

part of the toxin is taken across the vesicle membrane into the cytoplasm, where it finds its target and modifies it. In many cases, the very specific targets of these toxins are the crucially important cellular constituents. Toxins cover a wide sweep of biology and that is one of their fascinations.

12.6 Model Questions

1. Write an essay on different types of bacterial toxins with suitable examples.
2. Short answers.
    a. Superantigens
    b. Pore-forming toxins
    c. Shiga toxin
    d. Diphtheria toxin
    e. EPEC Tir
    f. *C. botulinum* exoenzyme 3

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Lesson No. 13

Basic characteristics and messengers of Cell Signaling

13.0 Objective

This lesson plan is aimed at giving the information about the extracellular first messenger and intracellular second messenger signal molecules that involves in various signal transduction pathways.

13.1 Introduction

No cell lives in isolation. In all multicellular organisms, survival depends on an elaborate intercellular communication network that coordinates the growth, differentiation, and metabolism of the multitude of cells in diverse tissues and organs. Cells within small groups often communicate by direct cell to cell contact. The establishment of specific cell-cell interactions between different types of cells is a necessary step in the development of many tissues. In some cases a particular protein on one cell binds to a receptor protein on the surface of an adjacent target cell, triggering its differentiation. The cells communicate by means of extracellular signaling molecules. These substances are synthesized and released by signaling cells and produce a specific response only in target cells that have receptors for the signaling molecules. An enormous variety of chemicals, including small molecules, peptides, and proteins, are used in this type of cell-to-cell communication. The extracellular products synthesized by signaling cells can diffuse away or be transported in the blood, thus providing a means for cells to communicate over longer distances than is possible by chains of direct cell-cell contacts. Internally in the cell this message is transduced to the effect by various second messengers like cAMP, cGMP, IP_3, DAG and Calcium ions.

13.2 Basic characteristics of cell-signaling systems

The term cell signaling conveys the concept that a cell responds to a stimulus from its environment by relaying information to its internal compartment. In most cases, the stimulus is a molecule that has been secreted into the extracellular space by another cell, but a stimulus may also originate from contact with another cell or a non-cellular substratum. Another term that is commonly used to describe this phenomenon is ‘Signal Transduction’, which indicates that the stimulus received by the cell-surface receptor is different from the signal released to the cell interior.

Signal transduction is a complex process in which information is passed along signaling pathways that consist of a series of distinct proteins. Each protein in the pathway typically acts by altering the conformation of the next protein in the series, an event that activates or inhibits that protein. The alterations in the conformation of signaling proteins

are usually accomplished by adding or removing phosphate groups. Signaling pathways contain protein kinases and phosphatases that induce rapid and reversible changes in cellular activities. Some of these enzymes have numerous proteins as their substrates, whereas others phosphorylate or dephosphorylate only a single protein substrate. Many of the protein substrates of these enzymes are other enzymes, but the substrates also include ion channels, transcription factors, and various types of regulatory subunits.

Another feature of cell-signaling pathways that is shared by other cellular processes is the involvement of GTP-binding proteins or G proteins as switches that turn activities on or off. G proteins can act as switches because they exist in two interchangeable states: an active state that contains a bound GTP and an inactive state that contains a bound GDP. In the active state, the GTP-bound protein binds to specific proteins i.e., downstream targets that the GDP-bound protein cannot bind. In most instances, binding of the GTP-bound protein activates the downstream target, allowing it to carry out a particular function. Hydrolysis of the bound GTP causes the G protein to revert to the inactive state, and it dissociates from the downstream target. The cycling of G proteins between active and inactive states is aided by accessory proteins that bind to the G protein and regulate its activity. These accessory proteins include –

- **GTPase-activating proteins (GAPs)** : Most G proteins possess some capability to hydrolyze a bound GTP, but this capability is greatly accelerated by the interaction with specific GAPs. Because of their ability to stimulate GTP hydrolysis and inactivate the G protein, GAPs dramatically shorten the duration of a G protein-mediated response.
- **Guanine nucleotide-exchange factors (GEFs)** : An inactive G protein is converted to the active form when the bound GDP is replaced with a GTP. GEFs are proteins that stimulate the dissociation of a bound GDP from the G protein. Once the GDP is released, the G protein rapidly binds a GTP, which is present at relatively high concentration in the cell, thereby activating the G protein.

- **Guanine nucleotide-dissociation inhibitors (GDIs)** : GDIs are proteins that inhibit the release of a bound GDP from a G protein, thus maintaining the protein in the inactive, GDP-bound state.

13.3 First Messengers

These first messengers are also called as Extracellular messengers or ligands. Different kinds of signaling molecules including proteins, small peptides, amino acids, nucleotides, bioactive amines, steroids, retinoids, fatty acid derivatives and even dissolved gases such as nitric oxide and carbon monoxide act as extracellular first messengers. Most of these signaling molecules are secreted from the signaling cell by exocytosis. Others are released by diffusion through the plasma membrane, while some remain tightly bound to the cell surface and influence only cells that contact the signaling cell.

Basing on their chemical nature, the first messengers can be grouped into –

Peptide messengers	---- Neuropeptides (neurotransmitter)
	Vasopressin (hormone)
Polypeptide messengers	---- Insulin (hormone)
Amino acid derivative messengers	---- Glycine (neurotransmitter)
	Epinephrine (hormone)
	Histamine (local mediator)
Fatty acid derivative messengers	---- Testosterone (hormone)
	Prostaglandins (local mediator)
Other small molecules	---- Acetylcholine (neurotransmitter)

13.3.1 Eicosanoids

These are the fatty acid derivatives and made by cells in mammalian tissues. They act in an autocrine mode in mature mammals. Eicosanoids are rapidly broken down and therefore act locally in autocrine and paracrine signaling pathways. There are four major classes of eicosanoids. All of them are made from arachidonic acid which in turn formed from phospholipids. These four classes are Prostaglandins, Prostacylins, Thromboxanes and Leukotrines. Of these, the synthesis of first three involves the enzyme

cyclooxygenase and the in the synthesis of leukotrines the enzyme lipoxygenase is involved. The important responses influenced by the eicosanoids in the target cells include – (i) platelet aggregation, (ii) inflammation and (iii) smooth-muscle contraction.

13.3.2 Steroid Hormones

These steroid hormones include cortisol, steroid sex hormones, vitamin D in vertebrates and ecdysone in insects. All of these are made from cholesterol. The ecdysone in insects trigger the metamorphosis of larvae to adults. These hormones bind to intracellular receptors that belong to the Steriod Receptor super family.

Cortisol: It is produced in cortex of adrenal gland and influence the metabolism of many cell types. It is a corticosteroid. The glucocorticoids act on a variety of cells to stimulate glucose production. The mineralocorticoids act on kidney to regulate salt and water balance.

Steroid sex hormones: These are made in testis and ovary and are responsible for the secondary sex characteristics that distinguish males from females. Testosterone, estrogen and progesterone are the sex hormones produced by gonads.

Vitamin D: It is synthesized in the skin in response to sun light. It is converted into an active form in liver or kidneys and function to regulate Ca^{2+} metabolism, promoting the Ca^{2+} uptake in the gut and reducing its excretion in the kidney.

13.3.3 Retinoids: These are the polyisoprenoid lipids derived from retinal (vitamin A). They perform multiple regulatory functions in diverse cellular processes. Retinoids regulate cellular proliferation, differentiation, and death, and they have numerous clinical applications. During the vertebrate development, retinoids act as local mediators of cell-cell interaction.

Though hormones are relatively insoluble in water, they are made soluble for tranport in the blood stream and other extracellular fluids by binding to specific carrier proteins, from which they dissociate before entering the target cell. In blood or tissue fluids, most water soluble hormones are removed or broken down within minutes after they entry into the blood. Local mediators and neurotransmitters are removed from extracellular space

even faster and within seconds or milliseconds. In contrast, the steroid hormones which are insoluble persist in blood for hours and thyroid hormones for days. Water soluble signal molecules mediate responses for short duration, whereas water insoluble signal molecules mediate responses for longer-lasting. The water soluble signaling molecules are the neurotransmitters, protein hormones and protein growth factors.

13.3.4 Nitric Oxide (NO)

NO is recently recognized as a signaling molecule in vertebrates. It is a paracrine signaling molecule in nervous, immune and circulatory systems. Like steroid hormones 'NO' diffuse through plasma membrane and it is unstable. NO is made by the enzyme 'NO synthase' by the deamination of the amino acid arginine. It acts locally as having a short half-life about 5-10 seconds, in the extracellular space before it is converted to nitrates and nitrites by oxygen and water. In target endothelial cells, NO reacts with iron that is bound to the active site of the enzyme 'guanylyl cyclase', stimulating it to produce the intracellular mediator cGMP which is a second messenger. Acetylcholine induce the endothelial cells to make and release the NO, which signals the smooth muscle cells to relax and blood vessel dilation. The use of nitroglycerine to treat patients with 'angina' is based on NO action. NO is also produced as a local mediator by activated macrophages and neutrophils to help them in killing invading microorganisms. The molecular basis of NO action distinct from steroid action, rather than binding to a receptor that regulates transcription, NO alters the activity of intracellular target enzymes.

Recently Carbon monoxide (CO) is also used as an intercellular signal in nervous system and it is known to act in the same way as NO, by stimulating guanylyl cyclase.

13.3.5 Cytokines

The term cytokine refers to a large, and steadily enlarging, group of proteins which act as cell-to-cell signaling agents and which are key controlling elements in the inflammatory response to infectious agents. There are different definitions for cytokines –

- Secreted regulatory proteins that control the survival, growth, differentiation and effector function of tissue cells

- A diverse group of soluble proteins and peptides which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues
- Polypeptide hormones secreted by a cell that affects growth and metabolism, either of the same cell (autocrine signaling) or of another cell (paracrine signaling)
- Proteins whose overproduction cause the disease

The first cytokine to be discovered was interleukin 1 (IL-1) and more than 100 distinct cytokines have been described. Structurally, cytokines are generally proteins of low molecular mass consisting of a single polypeptide side chain. Cytokines with structures other than a single polypeptide chain include TGFβ and IL-12. In addition, TNFα and TNFβ form homotrimers in solution. With a few exceptions, cytokines have no catalytic functions and must bind to a specific cell receptor in order to express biological activity. A large number of cytokine receptors have now been identified. The various cytokines can be subdivided into nine groupings which delineate the major biological actions of these proteins. These groups include –

- Proinflammatory IL-1, TNF, IL-6, IL-8, CSFs and chemokines
- Anti-inflammatory IL-4, IL-10, IL-12, IL-13and TGFβ
- B cell growth factors IL-4, IL-5, IL-6, IL-14
- Cytotoxic/growth inhibitors TNF, LT, Fas ligand
- Chemotactic factors IL-8, MCP-1
- Haematopoietic factors IL-3, M-CSF, G-CSF, GM-CSF
- Mesenchymal growth factors PDGF, TGFβs, EGF
- T cell growth factors IL-2, IL-4, IL-7, IL-9, IL-12
- Antiviral IFNs

13.4 Second Messengers

Binding of ligands to many cell surface receptors leads to a short-lived increase or decrease in the concentration of the intracellular signaling molecules termed as “Second Messengers”. These low molecular weight signaling molecules include –

- 3',5'-cyclic AMP (cAMP)
- 3',5'-cyclic GMP (cGMP)
- 1,2-diacylglycerol (DAG)
- inositol 1,4,5-triphosphate (IP_3)
- various inositol phospholipids (phosphoinositides)
- Ca^{2+}

The elevated intracellular concentration of one or more second messengers following hormone binding triggers a rapid alteration in the activity of one or more enzymes or non-enzymatic proteins. The various metabolic functions controlled by hormone-induced second messengers include –

- Uptake and utilization of glucose
- Storage and mobilization of fat
- Secretion of cellular products
- Proliferation, differentiation and survival of cells

13.4.1 Cyclic nucleotide second messengers

The earliest second messengers discovered are cyclic nucleotides cAMP and cGMP. The levels of these molecules in the cell are controlled by the opposing activities of 'nucleotidylate cyclases' which catalyze the reaction NMP → cNMP, and 'cyclic nucleotide phosphodiesterases (PDEs)' which catalyze the reverse reaction. Several early signaling pathways influence the activity of nucleotidylate cyclases. G-protein linked receptors of Gs and Gi families stimulate and inhibit adenylate cyclase, respectively, whereas transducin (Gt) stimulates cGMP PDE activity.

13.4.1.1 cAMP

The cAMP second messenger, for the first time, was identified by Sutherland. cAMP is a substance released into the interior of the cell following the binding of a first messenger- a hormone or any other ligand – to a receptor at the outer surface of the cell. It is one of the number of substances that act as second messengers in eukaryotic cells. This second messenger generated within the cytoplasm often stimulates a variety of cellular activities.

As a result, the second messengers enable cells to mount a large-scale, coordinated response following the stimulation by a single extracellular ligand. cAMP is synthesized by an integral membrane protein called 'adenylyl cyclase', whose catalytic domain resides at the inner surface of the plasma membrane. Adenylyl cyclase is called as 'effector', as it brings about or effects the cellular response by synthesizing the cAMP. The so formed cAMP diffuses into cytoplasm and evokes a response by initiating a chain of reactions. cAMP molecules bind to an allosteric site on a regulatory subunit of a specific cAMP-dependent protein kinase called protein kinase A (PKA). The PKAs are tetramers, consisting of two regulatory (R) subunits and two catalytic (C) subunits. In the tetrameric form, PKA is inactive. Binding of cAMP to the R subunits causes dissociation of the two C subunits in their active form, which then can phosphorylate specific acceptor proteins. A single hormone molecule that binds to cell surface can activate a number of adenylyl cyclase molecules, each of which can produce a large number of cAMP messengers in a short period of time. Production of second messenger is a major amplification step in the reaction cascade. Though the most rapid and best-studied effects of cAMP are produced in the cytoplasm, the nucleus and its genes also participate in the response. Though most of the activated PKA molecules remain in cytosol, a fraction of them translocate into the nucleus where they phosphorylate key nuclear proteins, most notably a 'transcription factor' called as cAMP response element-binding (CREB) protein. The phosphorylated version of CREB binds to sites on the DNA containing a particular nucleotide sequence known as the cAMP response element (CRE). The CREs are located in the regulatory regions of genes that play a role in the response to cAMP.

13.4.2 Lipid-derived Second Messengers

The phospholipids of cell membranes are converted into second messengers by activated phospholipases, which are hydrolytic enzymes that split phospholipids into their component parts. Phospholipid-derived second messengers are formed in a number of signaling pathways. The best-studied lipid-derived second messengers are derived from 'phosphatidylinositol', which are generated following the transmission of signals by G-protein coupled receptors. There is another group of second messengers which are derived from Sphingomyelin. The phospholipids involvement in cellular responses was

indicated first from the studies carried out in early 1950s by Lowell and Mabel Hokin. Addition of a single phosphate group to the inositol sugar of PI generates PI-4-phosphate (PIP), which is phosphorylated for second time to form PI-4,5-bisphosphate (PIP_2). When acetylcholine binds to a smooth muscle cell or an antigen binds to mast cell, the bound receptor activates a heterodimeric G protein which in turn activates the effector phosphatidylinositol-specific phospholipase C-β (PI-PLC β). The PI-PLC β is situated at the inner surface of the membrane. It catalyzes a reaction that splits PIP_2 into two molecules- inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Both of them play important roles as second messengers in cell signaling.

13.4.2.1 Diacylgylcerol (DAG)

DAG is a lipid molecule that remains in the plasma membrane following its formation by PI-PLC β. There it activates an effector, called 'Protein Kinase C' (PKC). This activated PKC phosphorylates serine and threonine residues on target proteins. PKC is a multifunctional serine and threonine kinase that phosphorylates a wide variety of proteins. PKC has a number of important roles in cellular growth and differentiation, cellular metabolism, and transcriptional activation. In liver cells, PKC works synergistically with PKA and both kinases phosphorylate glycogen synthase, inhibiting the activity of glycogen synthase. In myoblasts, the embryonic cells that give rise to muscle cells, the PKC activation leads to phosphorylation of the protein myogenin, a transcriptional factor that plays a key role in muscle cell differentiation. Phosphorylation of myogenin inhibits its ability to bind to DNA, thereby blocking the differentiation of the cells into muscle fibres. Various responses that are mediated by PKC include –

- Serotonin release in Blood platelets
- Histamine release in Mast cells
- Epinephrine secretion in Adrenal medulla
- Insulin secretion in Pancreas
- Calcitonin secretion in Thyroid
- Testosterone synthesis in Testis
- Dopamine release in Neurons

- Increased contractility in Smooth muscle
- Glycogen hydrolysis in Liver
- Fat synthesis in Adipose tissue

13.4.2.2 Inositol 1,4,5-trisphosphate (IP_3)

IP_3 is a small, water soluble, sugar phosphate molecule capable of rapid diffusion. The IP_3 molecules formed at the membrane diffuse into the cytosol and bind to a specific IP_3 receptor located at the surface of the smooth endoplasmic reticulum. The IP_3 on binding opens the receptor channel allowing the Ca^{2+} ions to diffuse into the cytosol. The Ca^{2+} ions released can also be considered as cellular messengers as they bind to various target molecules that trigger specific responses. Binding of vasopressin to its receptor at the liver cell surface causes a series of IP_3 – mediated bursts of Ca^{2+} release. Various cellular responses elicited by IP_3 include –

- Contraction of Vascular smooth muscle
- Contraction of Stomach smooth muscle
- Contraction of Skeletal muscle
- Shape change and aggregation of Blood platelets
- Increased potassium current in Lacrimal glands

13.4.3 Calcium Ions (Ca^{2+})

The calcium ion level in the cell plays a significant role in diverse processes including cell division, secretion, metabolism, muscle contraction, cell movement etc. The concentration of free calcium ions in different parts of a living cell can be measured by injecting the cell with fluorescent calcium-binding dyes and monitoring the light emitted using computerized imaging techniques. The level of Ca^{2+} in the cytoplasm is maintained at a low concentration by active export, but it is up to 10,000 fold higher in the extracellular fluid and in certain intracellular compartments. Depending upon the type of cell responding, a particular stimulus may induce oscillations in the level of free calcium ions.

The calcium ion is very different in structure from a cyclic nucleotide or an inositol phosphate and is not a substance that is synthesized or degraded enzymatically. Instead, the concentration of calcium ions in a particular cellular compartment is controlled by the regulated activity of Ca^{2+} transporters and Ca^{2+} channels located within the membranes that surround the compartment. The upstream signals originating at cell-surface receptors induce the release of Ca^{2+} into the cytoplasm by opening calcium channels. The calcium-binding proteins of cytosol activated by calcium ions, associate with inactive proteins and stimulate them to induce a number of downstream signaling pathways. Two major pathways stimulating calcium release are the G-protein linked receptors associated with Gq family proteins, and the RTKs. Both these receptors stimulate phospholipases which increase levels of Inositol 1,4,5 trisphosphate leading to the opening of Inositol 1,4,5-trisphosphate-gated Ca^{2+} channels in the membrane of endoplasmic reticulum.

13.5 Summary

Extracellular signaling molecules regulate interaction between unicellular organisms and are critical regulators of physiology and development in multicellular organisms. There are many different types of signals, including membrane-anchored and secreted proteins and peptides, small lipophilic molecules, small hydrophilic molecules derived from amino acids, and gases. Signals can act at short range, long range, or both. Various second messengers such as Ca^{2+}, cAMP, DAG and IP_3 are modulated in response to binding of ligand to cell-surface receptors. These intracellular signaling molecules, in turn, regulate the activities of enzymes and nonenzymatic proteins. Second messengers activate certain protein kinases. Binding of a second messenger leads to release of regulatory subunits of domains that mask the catalytic site(s), thereby activating the kinase activity.

13.6 Model Questions

1. Give an account on the kinds of second messengers.
2. Describe the basic characteristics of signal transduction and first messengers.
3. Short questions:
   (i) Cytokine first messengers

(ii) cAMP

(iii) Diacylglycerol

(iv) Calcium ions as second messengers

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Lesson No. 14

PROKARYOTIC CELL-TO-CELL SIGNALLING

14.0 Objective

This lesson plan study is aimed at knowing the mechanism of quorum sensing in communication between prokaryotic cells and intra cellular signaling mechanism involved in the chemotaxis.

14.1 Introduction

Mechanisms enabling one cell to influence the behaviour of another almost certainly existed in the world of unicellular organisms long before multicellular organisms appeared on earth. The concept that bacteria could talk to one another did not receive much attention until the 1980s when examples were found of cell-to-cell signaling in bacteria. The uses that bacterial cell-to-cell signaling mechanisms include – Sporulation and fruiting body formation in *Myxococcus xanthus*, Conjugation of *Enterococcus*

faecalis, Morphological differentiation in *Streptomyces coelicolor*, Antibiotic production by *Streptomyces* spp., and Autoinducer behaviour in many bacteria. Many of the mechanisms involved in chemical signaling between cells in multicellular animals have evolved from mechanisms used by unicellular organisms to respond to chemical changes in their environment. In fact, some of the same intracellular mediators, such as cyclic nucleotides, are used by both types of organisms. Among the best studied reactions of unicellular organisms to extra cellular signals are chemotactic responses, in which cell movement is oriented toward or away from a source of some chemical in the environment.

14.2 Bacterial pheromones and Quorum Sensing

14.2.1 Pheromones

Pheromones are the compounds that are released by organisms and that cause behavioral or physiological changes in other organisms of the same species. They are the communication chemicals used to send signals and allow for communication between members of the same species. Pheromones are often volatile compounds, since they have to be transmitted through the air. However, in some cases it may not be. Pheromones can also be transferred by direct physical contact between organisms. Also, pheromones are used by water organisms. Under water, volatility is of no importance, but good solubility is needed if the pheromones are to be spread over long distances. Different pheromones are used by different organisms, so that chemical communication can be used for different messages. The most important pheromones are sex pheromones and alarm pheromones, but there are numerous others, all are optimized for their specific tasks.

Bacterial pheromones, mainly different homoserine lactones, are central to a number of bacterial signaling processes, including those involved in plant pathogenicity. “N-oxoacyl-homoserine” lactone (OHL) is essential for quorum sensing in the soft-rot phytopathogen *Erwinia carotovora.* Quorum sensing, the ability of bacterium to communicate and coordinate via signaling molecules called autoinducers or pheromones, and also possess a receptor to detect the inducer. Quorum sensing was discovered by Bonnie Bassler and the first organism is *Vibrio fischeri*, a luminescent bacterium. The

purpose of quorum sensing is to coordinate certain behaviour or action between bacteria of the same kind, depending on their number.

14.2.2 Quorum Sensing

Quorum sensing is a bacterial cell-to-cell signaling system which has come to prominence in the last 10 years or so. The dictionary definition of the term quorum is 'a fixed number of members of any body, society etc., whose presence is necessary for the proper or valid transaction of business'. Bacterial quorum sensing is a mechanism by which bacteria can take a census of their numbers and having reached a 'quorum' can 'transact business' – only in the case of bacteria the business is the switching on or off of specific genes.

The current understanding of quorum sensing traces back to studies of the luminescence of the marine bacteria *Vibrio fischeri* and *V. harveryi*. These bacteria form symbiotic relationships with monocentrid fish and with species of squid known as bobtail squids (eg. *Euprymna scolopes*). Much of the understanding of the relationship between bacteria and marine organisms has come from the study of *Euprymna scolopes*. The bobtail squid has a light organ which contains very high concentrations of a monospecific culture of *V. fischeri*. The function of the light organ in the bobtail squid is thought to be part of a camouflaging behaviour called counter-illumination, but the details of this mechanism are still sparse. The adult light organ is bilobed, each lobe containing three epithelia lined crypts that house the bacteria. The light organ also has a pair of pores which allows it to make contact with the external environment. Newly hatched squids acquire their symbiotic bacteria from sea water, that is, transmission of the bacteria is horizontal and not vertical (from the parents). Sea water contains a large number of *Vibrio* species. In spite of this *Euprymna scolopes* is only colonized by *V. fishceri* and only certain strains at that. Thus the light organ appears to have a positive selection mechanism choosing only certain *V. fischeri* strains and a negative selection mechanism to exclude colonization by the vast numbers of other bacteria that are present in sea water. How this selection of bacteria is achieved is unclear. One obvious mechanism would be for the epithelium of the light organs to express a specific adhesin for *V. fischeri*. This idea is supported by

experiments in which the light organ epithelium is exposed to trypsin, which will remove surface peptides. Such proteolysis prevents colonization of the light organ by *V. fishceri*.

One of the most fascinating findings is that the adult light organ is only formed if the squid is infected with *V. fischeri*. Thus within hours after hatching the nascent light organ is infected or colonized by *V. fischeri*. In response the cells lining the crypts undergo terminal differentiation and the crypt spaces grow as a result of cell proliferation. However, if the animals are treated with antibiotics in the first 8-12 hours following colonization (which kills bacteria in the nascent light organ) the morphogenesis of the light organ does not occur. This suggests that *V. fischeri* produces signals which can directly trigger a specific morphogenetic response in the squid which results in the formation of the complex light organ.

14.2.3 Mechanism of Quorum Sensing

Quorum sensing is a remarkably simple feedback control system. It requires that the bacteria constantly produce small amounts of a signal called an autoinducer. The autoinducers produced by most Gram-negative bacteria are simple organic compounds known as acyl homoserine lactones (AHLs). Other bacteria, such as *Staphylococcus aureus*, produce peptide autoinducers. It ahs recently been reported that *E. coli* and *Salmonella typhimurium*, which were not thought to produce AHLs, also produce a quorum-sensing molecule of molecular mass 1 kDa which is sensitive to base and to heating to 100^{o}C. These autoinducers diffuse into the extracellular environment. The second requirement is that the bacterium has the means of recognizing the presence of the autoinducer. This function is served by a bacterial membrane protein which acts both as a receptor for the autoinducer and as an activator of gene transcription. This is related to the His-Asp phosphorelay systems of bacterial sensing and regulation.

The best-studied quorum-sensing system is the *V. fischeri* system that produces luminescence (Fig. 14.1). This comprises the lux genes which is an operon system involving two main regulatory genes and a number of other genes that synthesize the chemical reagents required to produce photons. The first regulatory gene is luxI and

encodes a protein which catalyses the synthesis of the AHL. This reaction uses S-adenosylmethionine as the donor of the homoserine lactone and a fatty acid moiety linked to an acyl carrier protein. A wide range of AHLs can be produced. For example, the autoinducer for *V. fischeri* is N-(3-oxohexanoyl)-L-homoserine lactone. The second regulatory gene is luxR and encodes a protein which acts both as receptor for AHL and as a transducer of the signal activating the other genes present in the lux operon. When AHL binds to the LuxR protein the genes LuxCDABEG are expressed. The luxA and luxB genes encode the α and β subunits of bacterial luciferase. The other genes encode polypeptides that are involved in the synthesis of the substrate (tetradecanal) used by the luciferase to generate light. This mechanism occurs daily in the bobtail squid. Every morning the squid expels 90-95% of the bacteria in its light organ and begins to repopulate these organisms over the next 12 hours, building up the huge numbers, 10^{10} bacteria / ml, required for its nocturnal lifestyle. It is at these astronomical numbers that the quorum sensing system is maximally stimulated and light is produced.

Fig. 14.1 – The luxI/luxR system of *Vibrio fishceri*

14.2.4 Quorum Sensing as a Virulence Mechanism

Since the discovery of the luxI / luxR system in *V. fischeri* a large number of Gram-negative bacteria have been reported to produce AHLs and presumably to quorum sense.

This list includes bacteria of medical importance such as *Pseudomonas aeruginosa*, *Y. enterocolitica*, *Proteus mirabilis*, *Serratia liquefaceins* and *Citrobacter freundii*. The luxI / luxR homologues are known to be involved in the quorum-sensing systems of these bacteria. The best studied pathogenic organisms, in terms of their quorum-sensing mechanisms, are *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The former is an opportunistic pathogen which causes particular problems to children with cystic fibrosis. Unlike *V. fischeri*, *Ps. aeruginosa* has evolved to utilize at least two quorum-sensing systems which have been termed 'las' and 'rhl'. The las operon encodes the LasR protein, a LuxR homologue which acts as a transcriptional activator in the presence of autoinducer for this bacterium. This AHL is produced by the LuxI homologue, LasI. When concentrations of the *Ps. aeruginosa* autoinducer reach a threshold they switch-on a collection of virulence genes including lasB, lasA, apr and toxA. The second quorum-sensing system is the rhl system, which consists of the transcriptional activator protein RhlR with the autoinducer bering N-butyryl-L-homoserine lactone, which is synthesized by RhlI. This quorum-sensing system is involved in the production of additional virulence factors such as the well-known elastase that is capable of cleaving and inhibiting key host defence cytokines such as interleukin 2. Having two quorum-sensing systems opens up the possibility that they may interact, and this is precisely what they do. The las system appears to be dominant and *Pseudomonas* autoinducer (PAI) can act to inhibit the binding of the second autoinducer (N-butyryl-L-homoserine lactone) to the LuxR homologue RhlR. Thus there is a hierarchy in the quorum sensing of this bacterium to ensure that the las system is activated before the rhl system. The reason for such a hierarchical control is not yet clear and awaits a complete explanation.

Several other bacteria quorum sense but do so using signals distinct form the AHLs. Several Gram-positive bacteria use oligopeptides as signaling molecules. *Bacillus subtilis* secretes at least two different peptides which are necessary for competence (ability to take up DNA) and sporulation. The opportunistic pathogen *Staphylococcus aureus* has a locus termed agr which controls the expression of several virulence factors such as exotoxins, the V8 protease and capsular polysaccharide type 5. The agr locus encodes an octapeptide which is believed to be a quorum-sensing autoinducer which activates the agr

locus. The study of quorum sensing is still in its early phase and much remains to be learned. One recent finding is that the quorum-sensing autoinducer from *Ps. aeruginosa* can interact with host defence systems and can inhibit them, albeit at high concentrations. This ability of quorum-sensing autoinducers to both activate bacterial virulence factors and inhibit host defence mechanisms is the equivalent of an evolutionary 'double whammy'.

14.3 Signals controlling conjugation in *Enterococcus faecalis*

One of the three natural mechanisms of transfer of DNA between bacteria is bacterial conjugation, which requires that bacteria come into contact with one another. In *Enterococcus faecalis*, a Gram-positive member of the mammalian commensal microflora, the aggregation of bacteria is controlled by the secretion of peptide pheromones. These small peptides induce the production of the adhesions which enable bacteria to form clumps or cellular aggregates within which conjugation takes place. A number of peptide pheromones have been isolated and they are generally hydrophobic octapeptides or heptapeptides. These pheromones are active at extremely low molar concentrations and it has been calculated that as few as two molecules of the peptide per cell many be sufficient to produce biological activity. In addition to their potency these pheromones demonstrate marked selectivity. This agonist-receptor system appears to have significant similarities to the cytokine signaling systems of eukaryotes in terms of potency and selectivity.

14.4 Signals controlling sporulation in *Myxococcus xanthus*

Sporulation is a fairly common response of bacteria to adverse conditions. Endospores are highly resistant resting forms of bacteria that are produced within the cell rather than on specialized external structures. The endospores of *Clostridium tetani* can be regarded as part of the virulence mechanism of this organism and leads to us being constantly on guard against injuries involving soil which contains such endospores. Now, in contrast to endospores, some bacteria respond to adverse conditions by undergoing complex morphogenetic alterations and forming what are known as fruiting bodies: cyst-like structures composed of an outer thick covering of polysaccharide which makes the cyst

resistant to heat and dehydration. The visibility of these fruiting bodies accounts for the fact that it was a myxobacterium-*Polyangium vitellinum*- that was the first prokaryote to be assigned a scientific name in 1809. The best studied sporulation system is that of the Gram-negative soil bacterium *Myxococcus xanthus*. This organism undergoes a complex life cycle which alternates between the formation of myxospores (fruiting body) and of what are termed vegetative cells. This developmental programme is triggered by starvation and leads to morphological changes within 4 hours as the vegetative cells begin to congregate. When a cell density of about 10^5 organisms is reached, a dense mound-shaped structure is formed that is just visible to the unaided naked eye. After 20 hours of starvation cells inside this mound differentiate into myxospores that are heat- and desiccation-resistant dormant cells and form a fruiting body. When favourable conditions are again encountered the fruiting body 'germinates', producing vegetative cells which are tapered and flexible. These cells grow and divide and produce swarms of individual cells whose activity has been likened to that of 'wolf-packs' moving over a solid surfaces and devouring bacteria encountered in their paths. When conditions again become unfavourable the formation of the myxospores begins anew. This complex cell differentiation is controlled by extracellular signals. This was discovered when mutants unable to sporulate were incubated with wild-type organisms and regained the ability to form myxospores. A number of signals appear to be involved in this bacterial differentiation system including small peptides and a 17 kDa protein.

14.5 Chemotaxis and Signal Transduction

The movement of bacteria in response to the concentration of a chemical substance is referred to Chemotaxis. The movement of motile bacteria toward higher concentrations of nutrients or attractants such as sugars, amino acids, and small peptides is known as positive chemotaxis. The motility of bacteria away from higher concentrations of various noxious chemicals or repellents is called as negative chemotaxis. This relatively simple but highly adaptive chemotactic behaviour has been mist studied in *Escherichia coli* and *Salmonella typhimurium*. Chemotaxis away from repellents depends on essentially the same mechanisms operating in reverse to that of chemotaxis toward attractants.

Bacteria swim by means of flagella that are completely different from the flagella of eukaryotic cells. The bacterial flagellum consists of a helical tube formed from a single type of protein subunit, called flagellin. Each flagellum is attached by a short flexible hook at its base to a small protein disc embedded in the bacterial membrane. This disc is part of a tiny "motor" that uses the energy stored in the transmembrane H^+ gradient to rotate rapidly and turn the helical flagellum. Because the flagella on the bacterial surface have an intrinsic "handedness", different directions of rotation have different effects on movement. Counterclockwise rotation allows all the flagella to draw together into a coherent bundle so that the bacterium swims uniformly in one direction. Clockwise rotation causes them to fly apart, so that the bacterium tumbles chaotically without moving forward. In the absence of any environmental stimulus, the direction of rotation of the disc reverses every few seconds, producing a characteristic pattern of movement in which smooth swimming in a straight line is interrupted by abrupt, random changes in direction caused by tumbling.

The normal swimming behaviour of bacteria is modified by chemotactic attractants or repellents, which bind to specific receptor proteins and affect the frequency of tumbling by increasing or decreasing the time that elapses between successive changes in direction of flagellar rotation. When bacteria are swimming in a favourable direction (toward a higher concentration of an attractant or away from a higher concentration of a repellent), they tumble less frequently than when they are swimming in an unfavourable direction (or when no gradient is present). Since the periods of smooth swimming are longer when a bacterium is traveling in a favourable direction, it will gradually progress in that direction i.e. toward an attractant or away from a repellent.

In its natural environment a bacterium detects a spatial gradient of attractants or repellents in the medium by swimming at a constant velocity and comparing the concentration of chemicals over time. Changes over time can be produced artificially in the laboratory by the sudden addition or removal of a chemical to the culture medium. When an attractant is added in this way, tumbling is suppressed within a few tenths of a second, as expected. But after some time, even in the continuing presence of the

attractant, tumbling frequency returns to normal. The bacteria remain in this adapted state as long as there is no increase or decrease in the concentration of the attractant; addition of more attractant will briefly suppress tumbling, whereas removal of the attractant will briefly enhance tumbling until the bacteria again adapt to the new level. Adaptation is a crucial part of the chemotactic response in that it enables bacteria to respond to changes in concentration.

The unraveling of the molecular mechanisms responsible for bacterial chemotaxis has depended largely on the isolation and analysis of mutants with defective chemotactic behaviour. Chemotaxis to a number of chemicals depends on a small family of closely related transmembrane receptor proteins that are responsible for transmitting chemotactic signals across the plasma membrane. These chemotaxis receptors are methylated during adaptation and so are also called *methyl-accepting chemotaxis proteins* (MCPs). The receptor activity is stimulated by an increase in repellent concentration and decreased by an increase in attractant concentration: a single receptor is affected by both sorts of molecules, with opposite consequences. There are four types of plasma membrane chemotaxis receptors, each concerned with the response to a small group of chemicals. Type 1 and 2 receptors mediate responses to serine and aspartate, respectively, by binding these amino acids and transducing the binding event into an intracellular signal. Type 3 and 4 receptors mediate responses to sugars and dipeptides, respectively, in a slightly less direct fashion.

Genetic studies indicate that four cytoplasmic proteins – *CheA, CheW, CheY, and CheZ* – are involved in the intracellular signaling pathway (Fig. 14.2) that couples the chemotactic receptors to the flagellar motor. CheY acts at the effector end of the pathway to control the direction of flagellar rotation. When activated, it binds to the motor, causing it to rotate clockwise and thereby inducing tumbling; mutants that lack this protein swim constantly without tumbling. CheA is a histidine protein kinase. When bound to both an activated chemotactic receptor and CheW, it phosphorylates itself on a histidine residue and almost immediately transfers the phosphate to an aspartic acid residue on CheY. The phosphorylation of CheY activates the protein so that it binds to

the flagellar motor and causes clockwise rotation and tumbling. CheZ rapidly inactivates phosphorylated CheY by stimulating its dephosphorylation.

The binding of a repellent to a chemotactic receptor increases the activity of the receptor, which in turn increases the activity of CheA and thereby the phosphorylation of CheY, which causes tumbling. These phosphorylations occur rapidly: the time required for the tumbling response after adding a repellent is about 200 milliseconds. The binding of an attractant has the opposite effect. It decreases the activity of the receptor , which decreases the activity of CheA, so that CheY remains dephosphorylated, the motor continues to rotate counterclockwise, and the bacterium swims smoothly. The function of CheY in bacterial chemotaxis is analogous to the function of Ras proteins in animal cell signaling. Like Ras, CheY functions as an on / off switch: it is on when phosphorylated and off when dephosphorylated , just as Ras is on with GTP bound and off with GDP bound. CheY is activated by CheA and inactivated by CheZ, just as Ras is activated by GNRPs and inactivated by GAPs. Indeed, the three-dimensional structures of CheY and Ras are similar.

Adaptation in bacterial chemotaxis results from the covalent methylation of the chemotaxis receptor proteins. When methylation is blocked by mutation, adaptation is markedly inhibited, and exposure of the mutant bacteria to an attractant results in the suppression of tumbling for days instead of for a minute or so. Binding of a chemoattractant to a chemotaxis receptor, therefore, has two separable consequences: (1) it rapidly decreases the activity of the receptor, thereby decreasing the activity of CheA and CheY and causing the flagellar motor to continue to rotate counterclockwise; this results in a suppression of tumbling. (2) it causes adaptation because, while the attractant is bound, the receptor is methylated by an enzyme in the cytoplasm, which increases the activity of the receptor over a period of a few minutes.

Receptor methylation is catalyzed by an enzyme (methyl transferase) that acts on the receptor protein. As many as eight methyl groups can be transferred to a single receptor, the extent of methylation increasing at higher concentrations of attractant (where each

receptor spends a larger proportion of its time with ligand bound). When the attractant is removed, the receptor is demethylated by a demethylating enzyme (methylesterase). Although the level of methylation changes during a chemotactic response, it remains constant once a bacterium is adapted because a balance is reached between the rates of methylation and demethylation. The methylesterase that removes methyl groups from the chemotactic receptors is also regulated by CheA-mediated phosphorylation, and this provides another form of negative feedback regulation that makes a further contribution to adaptation.

Fig. 14.2- Signal transduction and phosphorelay system in chemotaxis

14.6 Summary

Communication is paramount in biology and many varieties of signals are used to communicate between cells. It has come as a surprise to find that bacteria also utilize complex cell-to-cell and intracellular signaling pathways to communicate with each other, and also with eukaryotic cells, and the similarity of the mechanisms used by both prokaryotes and eukaryotes is striking. One of the major discoveries of cellular microbiology is that bacteria have the capacity to utilize eukaryotic cell-signaling

pathways during the process of infection. By adapting to high concentrations of a signaling ligand in a time-dependent, reversible manner, cells can adjust their sensitivity to the level of stimulus and thereby respond to changes in a ligand's concentration over an enormously large range rather than to the absolute concentration of the ligand. The adaptation occurs in various ways. At a molecular level the best-understood example of adaptation occurs in bacterial chemotaxis, in which the reversible methylation of key signal-transducing proteins in the plasma membrane helps the cell to swim toward an optimal environment.

14.7 Model Questions

1. What is quorum sensing? Describe the mechanism of quorum sensing in bacteria with suitable example.
2. Define chemotaxis? Explain the signal transduction process in chemotaxis of bacteria.

14.8 Reference Books

1. Cossart, P., Boquet, P., Normark,S. and Rappuoli, R (2000) – Cellular Microbiology – ASM Press, Washington, USA

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Lesson No. 15

Eukaryotic Cell-to-Cell Signaling

15.0 Objective

The objective of this lesson plan is to explain the two important signaling mechanisms viz., hormone signaling and cytokine signaling with selected examples.

15.1 Introduction

Cells, to survive, must communicate with their neighbours, monitor the environmental conditions and respond to their environment by reorganizing their structure, regulating the activity of proteins and altering patterns of gene expression. The stimulus for such responses is termed as 'signal' and the phenomenon by which cells carryout these interactions is called as 'Cell Signaling' or 'Signal Transduction'. A signal may be a small molecule, a macromolecule or a physical agent such as light. These signals interact with the responding cell through molecules termed as 'Receptors'. A molecular signal that binds to a receptor is termed as 'Ligand'.

In most of the systems, cell signaling includes –

- Recognition of the stimulus at the outer surface of the plasma membrane by a specific receptor embedded in the membrane.
- Transfer of a signal across the plasma membrane to its cytoplasmic surface.
- Transmission of the signal to specific effector molecules on the inner surface of the membrane or within the cytoplasm that trigger the cell's response. Depending on the type of cell and stimulus, the response may involve a change in gene expression, an alteration of the activity of metabolic enzymes, reconfiguration of the cytoskeleton, change in ion permeability, activation of DNA synthesis and even cell death.
- Cessation of the response occurs as a result of the destruction or inactivation of the signaling molecule, combined with a decrease in the level of the extracellular stimulus.

Cell signaling mechanism, enabling one cell to influence the behaviour of another cell existed in the world of unicellular organisms and long before in multicellular organisms. Evidence is available from the present-day unicellular eukaryotes such as yeasts. Though yeast cells normally lead independent lives, they can communicate and influence one another's proliferation in preparation for sexual mating. In budding yeast "*Saccharomyces cerevisiae*", when a haploid individual is ready to mate, it secretes a peptide 'mating factor' that signals cells of opposite mating types to stop proliferating and prepare to conjugate; the subsequent fusion of two haploid cells of the opposite mating type produces a diploid cell, which can then undergo meiosis and sporulate to generate haploid cells with new assortments of genes.

In most of the cases, stimulus comes from the molecules secreted by a cell into the extracellular space. But stimulus may also originate from contact with another cell or a non-cellular substratum. Signaling molecules that a cell secretes may be carried far afield to at on distant targets, or they may act as local mediators affecting only cells in the immediate environment of the signaling cell.

Signals may be processed in three ways –

- Certain chemical signals may penetrate the plasma membrane of the cell and interact with internal receptors. Eg: Steroids, Nitric Oxide
- Most signals are hydrophilic molecules remaining outside the cell. These interact with transmembrane or membrane associated receptors and cause a change of receptor structure. This interaction may result in signal transport, i.e., signaling molecule is internalized. Internalization may be through conformation change of the receptor, a pore created as in the case of ion channels, or by receptor-mediated endocytosis.
- Alternatively, the conformational change in the receptor may induce enzyme activity inside the cell which mediates downstream effects while ligand remains on the outside.

Signal transduction involves pathways of sequential enzyme activation and modulation of the levels of small molecules termed as second messengers. This allows the amplification of the original signal. Signal transduction pathways can converge and diverge, allowing multiple stimuli to generate similar responses, and individual signals to effect different responses. Further diversity is generated by different responses to the length and intensity of the stimulus, and the cell-specific synthesis of different receptors and signaling components.

In some cases of signals, a single signaling molecule often has different effects on different target cells. For example, the neurotransmitter ‘acetylcholine’ stimulates the contraction of skeletal muscle cells but decreases the rate and force of contraction in heart muscle cells. This may be because of receptor proteins for acetylcholine on skeletal and muscle cells are different. But in many cases, the same signaling molecule binds to identical receptor proteins and yet produces different responses in different types of target cells, reflecting differences in the internal machinery to which receptors are coupled.

15.2 Major forms of Eukaryotic cell-to-cell signaling systems

In multicellular organisms like that mammals three major forms of signaling systems are used –

Neuronal or Synaptic Signaling: When activated by signals from the environment or from other nerve cells, a neuron sends electrical impulses along its long processes called "axons". When an impulse reaches the nerve terminals at the end of the axon, is stimulates the terminals to secrete a chemical signal called 'neurotransmitter'. The nerve terminals contact their target cell at specialized cell junctions called 'chemical synapses', which are designed to ensure that the neurotransmitter is delivered to the post synaptic target cell rapidly and specifically. This signaling is a rapid mode of communication, which can occur over very long distances.

Endocrine Signaling: Endocrine cells are the specialized cells that control the behaviour of the organism. They secrete their signaling molecules called as 'hormones', into the blood stream, which carries the signal to the target cells distributed widely throughout the body. For example, hormone from glandular tissue transport through blood vessel to reach limited number of cells in target tissue. This signaling can occur over long distances, but slow due to limitation as rate of blood flow and diffusion.

Cytokine Signaling: This is the most recently discovered signaling mechanism. Much of the signaling occurs over short distances either cell to nearby cell (paracrine signaling) or by stimulation of the cell producing the cytokine (autocrine signaling).

15.3 Four Major Classes of Cell-Surface Receptors

The different types of cell-surface receptors that bind and interact with ligands can be sorted into four classes (Fig. 15.1). These are –

- **G Protein-coupled receptors** : Ligand binding activates a G protein, which in turn activates or inhibits an enzyme that generates a specific second messenger of modulates an ion channel, causing a change in membrane potential. The receptors for epinephrine, serotonin, and glucagons are the examples.

- **Ion-channel receptors**: Ligand binding changes the conformation of the receptor so that specific ions flow through it; the resultant ion movements alter the electric potential across the cell membrane. The acetylcholine receptor at the nerve-muscle junction is an example.

- **Tyrosine kinase-linked receptors**: These receptors lack intrinsic catalytic activity, but ligand binding stimulates formation of a dimeric receptor, which then interacts with and activates one or more cytosolic protein-tyrosine kinases. The receptors for many cytokines, the interferons, and human growth factor are of this type. These tyrosine kinase-linked receptors sometimes are referred to as the cytokine-receptor super family.

- **Receptors with intrinsic enzymatic activity:** Several types of receptors have intrinsic catalytic activity, which is activated by binding of ligand. For instance, some activated receptors catalyze conversion of GTP to cGMP; others act as protein phosphatases, removing phosphate groups from phosphotyrosine residues in substrate proteins, thereby modifying their activity. The receptors for insulin and many growth factors are ligand-triggered protein kinases; in most cases, the ligand binds as a dimmer, leading to dimerization of the receptor and activation of its kinase activity. These receptors – often referred to as receptor serine/threonine kinases or receptor tyrosine kinases – autophosphorylate residues in their own cytosolic domain and also can phosphorylate various substrate proteins.

Fig. 15.1 Four classes of ligand-triggered cell-surface receptors

15.4 Hormone Signaling

15.4.1 G protein-coupled receptors' (GPCRs) activation

Glucagon is a small protein of 29 amino acids; epinephrine is a small hydrophilic amine that is synthesized from the amino acid tyrosine. These two molecules have virtually nothing in common structurally, yet both of them activate the same effector (adenylyl cyclase) leading to the synthesis of cyclic AMP and the subsequent phosphorylation of phosphorylase. The receptors for these two very different ligands are members of the same family of integral membrane proteins, which are characterized by seven membrane-spanning α helices. The transmembrane segments are connected to one another by short extracellular and intracellular loops. The two receptors differ from one another primarily in the structure of the ligand-binding pocket on the extracellular surface of the receptor, which is specific for one or the other hormone.

Not only are the two receptors very similar in structure, so too are the proteins that transmit the signal from the ligand-bound receptor to the adenylyl cyclase effector. Transmission of the signal from receptor to effector is accomplished by a third component of the transduction system, a heterotrimeric G protein, which is held at the plasma membrane by covalently linked lipid chains. These proteins are referred to as G proteins because they bind guanine nucleotides, either GDP or GTP. They are described as heterotrimeric because all of them consist of three different polypeptide subunits, called α, β, and γ. Seven-helix transmembrane receptors and heterotrimeric G proteins are components of a widespread mechanism of signal transduction in eukaryotic cells.

Hundreds of different GPCRs have been identified in organisms ranging from yeast to flowering plants and mammals. These receptors respond to a diverse array of hormones, neurotransmitters, opium derivatives etc. Activation of G protein-coupled receptors follows a similar sequence of events regardless of the particular stimulus and activate the adenylyl cyclase (Fig. 15.2). This sequence include the steps viz., --

- **Activation of the G protein by the receptor:** When a ligand binds to one of these receptors, it induces a change in the conformation of the receptor that increases its affinity for the G protein. This is the sole function of the

extracellular ligand. The ligand-bound receptor binds to the G protein on the inner surface of the membrane, forming a receptor-G protein complex. Interaction with the receptor causes the α subunit of the G protein to release its bound GDP? And bind a GTP replacement, which switches the G protein into the active state. While in the activated state, a single receptor can activate a succession of G protein molecules, providing the first amplification step in the pathway.

- **Relay of the signal from G protein to effector:** The exchange of GDP by GTP alters the conformation of the G_α subunit, causing it to dissociate from the receptor and from the other two subunits of the G protein, which remain together as a $G_{\beta\gamma}$ complex. Each dissociated G_α subunit is free to activate an effector molecule, such as adenylyl cyclase, which puts the second messenger system into operation. As long as a G_α-GTP remains bound to an adenylyl cyclase molecule, the enzyme will continue to produce cAMP molecules. The $G_{\beta\gamma}$ subunit complex may activate other downstream effectors, providing an additional pathway for transmission of signals in a target cell.

- **Ending the response:** Signaling is terminated when the GTP molecule bound to the G_α subunit is hydrolyzed to GDP. Thus, the strength and duration of the signal is determined in part by the rate of GTP hydrolysis. G_α subunits possess a weak GTPase activity, which allows them to slowly hydrolyze the bound GTP and inactivate themselves. Termination of the response is accelerated by interaction with an accessory protein that increases the efficiency of the GTPase catalysis by the G_α subunit. Once the GTP is hydrolyzed, the G_α-GDP can reassociate with the $G_{\beta\gamma}$ subunits to reform the inactive trimeric complex and return the system to the resting state.

Fig. 15.2 Activation of adenylyl cyclase following binding of an hormone to GPCR

15.4.2 Receptor Tyrosine Kinases (RTKs) and Ras

The receptor tyrosine kinases are the second major type of cell-surface receptors. The ligands for RTKs are soluble or membrane-bound peptide/protein hormones including nerve growth factor (NGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and insulin. Binding of a ligand to this type of receptor stimulates the receptor's intrinsic protein tyrosine kinase activity, which subsequently sitmulates a signal-transduction cascade leading to changes in cellular physiology and / or patterns of gene expression. RTK signaling pathways have a wide spectrum of functions including regulation of cell proliferation and differentiation, promotion of cell survival, and modulation of cellular metabolism.

Insulin acts on liver and muscle cells to initiate an opposite series of reactions in which glucose is removed from the bloodstream and polymerized as glycogen. In addition to its effects on glucose uptake and metabolism, insulin is a powerful stimulant of lipid synthesis in fat cells, protein synthesis as well as cell growth and proliferation. Insulin elicits certain of these responses by a signaling pathway that is different in many respects from that used by glucagons and epinephrine.

Cells that respond to insulin possess an insulin receptor at their surface. The insulin receptor is more than just a protein that binds a ligand; it is also an enzyme – a protein tyrosine kinase – that adds phosphate groups to specific tyrosine residues of other proteins. Tyrosine kinases are involved primarily in the control of cell growth and differentiation, rather than the control of intermediary metabolism. Because the insulin receptor has this enzymatic activity, it is referred to as **receptor tyrosine kinase** (or **RTK**). Unlike the G protein-coupled receptors, which have seven transmembrane segments, each RTK monomer traverses the membrane only once.

The insulin receptor is a tetrameric protein composed of two α and two β polypeptide chains linked by disulfide bonds. The α chains reside on the extracellular surface of the membrane and contain the insulin-binding sites, whereas the β chains span the membrane and transmit the signal across the membrane to its inner surface. In the absence of bound

insulin, the tyrosine kinase function of the receptor is inactive. Binding of insulin to the receptor's α subunit changes the conformation of the β subunit, activating its tyrosine kinase. The activated tyrosine kinase transfers phosphate groups to (i) specific tyrosine residues of the other β subunit of the complex, a reaction termed autophosphorylation, and (ii) a dozen or more tyrosine residues of at least two protein substrates, called **insulin receptor substrates** (IRSs). Phosphorylated IRSs bind and activate a variety of downstream effectors.

RTKs do not phosphorylate every tyrosine in a substrate protein; they phosphorylate only those that are present within certain amino acid sequences referred to as *phosphotyrosine motifs.* A variety of proteins involved in cell signaling contain modules, called SH2 domains, that possess high-affinity binding sites for phosphotyrosine motifs. SH2 domains have little or no affinity for proteins whose tyrosine residues are not phosphorylated. It is only after an IRS has been phosphorylated by the insulin receptor that the IRS acts as a magnet to bind proteins that possess SH2 domains. The consequence of this interaction depends on the particular proteins involved. The interaction may turn on an enzymatic activity of the SH2 protein; change the protein's conformation, causing it to bind other proteins; or cause the protein to be translocated to a different part of the cell. Once the insulin receptor has been activated, the phosphorylated IRS proteins serve as 'docking sites' for a number of different SH2-contianing proteins, each of which may activate a separate signaling pathway. As a result, the message that insulin has bound to the cell surface may radiate through the cell interior along several independent pathways.

The steps in the Ras signaling pathway that follow binding of a growth factor like EGF to an RTK results in the generation of phosphotyrosines in the cytoplasmic domain of the RTK by autophosphorylation. These locations of phosphotyrosines act as binding sites for a specific SH2 protein called Grb2 . Grb2 is ot a protein with catalytic activity, but one that functions solely as an adaptor molecule that links other proteins into a complex. The structure of Grb2 is consisting of three distinct domains, each of which binds to another protein. One domain of the Grb2 molecule binds to the phorphorylated RTK at

the inner surface of the membrane, while another domain binds to a protein called Sos. Sos is a guanine nucleotide exchange factor for Ras, that is a Ras-GEF. The GEFs activate G proteins by stimulating the exchange of GDP with GTP.

In the unstimulated cell, Ras remains bound to GDP. When a ligand binds to the RTK and recruits the Grb2-Sos to the inner surface of the membrane, the Sos protein binds to Ras, causing it to lose its GDP, which is replaced by GTP, thus activating Ras. The primary function of Ras-GTP is to recruit another protein, called Raf, to the plasma membrane. Once it is localized at the plasma membrane, Raf becomes activated as a protein kinase that initiates an orderly chain of phosphorylation reactions called the **MAP kinase cascade**. The MAP kinase cascade is similar to the cascade of reactions triggered by cAMP during glucose mobilization, but even more complex. Once activated, the last protein kinase in the cascade (MAPK) translocates to the nucleus where it phosphorylates and activates specific transcription factors, such as Elk-1. Elk-1 binds to the promoter regions of a number of genes, including c-fos and c-jun. The products of these genes, Fos and Jun, interact to form a heterodimeric transcription factor called AP-1 that activates genes involved in the cell proliferation. Dimerization of RTKs and Ras activation on binding of hormone to an RTK are given in figures 15.3 and 15.4, respectively.

Fig. 15.3 – General structure and activation of RTKs

Fig. 15.4 – Activation of Ras following binding of a hormone to an RTK

15.5 Cytokine Signal Transduction

In general, binding of any cytokine to its cellular receptor triggers intracellular kinase activity. This can either be a direct effect of the receptor, which may have its own kinase domain, or it may require the formation of complexes with other intracellular proteins to

form a kinase domain. The aim of this to produce selective signals which can enter into the nucleus of the cell and induce the transcription of particular genes. In the past few years one particular signaling system has been discovered which is beginning to explain how it is possible that cytokines can have the amazing range of actions on cells that they do. This signaling system occurs when cytokines bind to either class I or class II receptors. Having bound to the receptor and induced dimerization of the particular receptor-signaling protein complex, the intracytoplasmic domain of the dimerized receptor complex is phosphorylated on tyrosine residues by a family of bound tyrosine kinases, the Jak (or JAK) tyrosine kinases. This phosphorylation induces binding sites for the SH2 binding domains of particular proteins. In this case the proteins are a group of latent cytosolic transcription factors known as 'Signal Transducers and Activators of Transcription (STATs). There are seven STAT proteins (STAT 1-4, STAT 5a, STAT 5b and STAT 6). The STATs bind to the dimerized receptor where they are phosphorylated by the JAKs. This allows them to dissociate and form homo- or hetero-dimers with other STATs, thus forming DNA binding complexes which act as specific transcription activators. It appears that some of the STATs may confer specificity on the signaling induced by certain cytokines.

For example, the binding of IL-6 to its respective receptor causes the dimerization of the gp 130 signaling proteins required to transduce the IL-6 binding event (Fig. 15.5). The dimerization of gp 130 causes the activation of the JAK kinases which associate with the intracellular domain of this protein. This causes tyrosine phosphorylation of the distal part of gp 130. STAT 3 recognizes and binds to this P-Y motif via its src homology-2 or SH2 domain and is phosphorylated by the JAK kinases. The tyrosine-phosphorylated STAT 3 proteins form homodimers and translocate to the nucleus and cause the transcription of specific target genes.

Fig. 15.5 – Cytokine signaling via the JAK-STAT mechanism

15.6 Summary

Many cell-surface receptors are linked to trimeric G proteins. Ligand binding to these receptors leads to activation of an associated signal-transducing G protein. Binding of ligand to a G protein-coupled receptor causes a conformational change that permits the receptor to bind to a specific G protein. Adenylyl cyclase, which catalyzes the formation of cAMP from ATP, is the best characterized effector regulated by trimeric G proteins.

Receptor tyrosine kinases (RTKs), which bind to peptide/protein hormones, may exist as dimers or dimerize during binding to ligands. Ligand binding leads to activation of the kinase activity of the receptor and autophosphorylation of tyrosine residues in its cytosolic domain. The activated receptor also can phosphorylate other protein substrates. Ras is an intracellular GTPase switch protein that acts downstream from most RTKs. Unlike GPCRs, which interact directly with an associated G protein, RTKs are linked indirectly to Ras via two proteins, GRB2 and Sos. Binding of Sos to inactive Ras causes a large conformational change that permits release of GDP and binding of GTP. Normally, Ras activation and the subsequent cellular response is induced by ligand binding to an RTK. However, in cells that contain a constitutively active Ras, the cellular response occurs in the absence of ligand binding.

15.7 Model Questions

1. Explain the GPCRs activation signal mechanism in eukaryotes
2. Describe the RTKs and Ras signal transduction pathway
3. Give an account on the cytokine signal transduction mechanism.

15.8 Reference Books

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Lesson No. 16

APOPTOSIS

16.0 Objective

To knowledge the reader about the genetically programmed cell death process in eukaryotes and the role of some pathogenic bacteria in inducing the apoptotic program in their respective hosts.

16.1 Introduction

Cells can self-destruct via an intrinsic program of self death. There are two distinct mechanisms of cell death. One is apoptosis and the other is necrosis. The most significant difference between them is that in apoptosis the cell's own molecules are ultimately responsible for its death while in necrosis the cell is a victim of molecules

synthesized by other cells. For example, apoptosis can be initiated by a cytokine binding to its receptor, which initiates a signaling cascade that activates a cell death program. Necrosis results form the effects of toxic molecules on a cell. In multicellular organisms both development and homeostasis are achieved through a balance between cell death and cell growth.

Apoptosis is activated in multicellular organisms when death is desirable for the well-being of the whole organism. A classic and graphic example of the importance of apoptosis in development can be seen during the morphogenesis of the limb. During embryogenesis, the digits are interconnected by a web of cells. At a precise time point, the cells that constitute the interdigital web undergo programmed cell death, allowing the formation of independent fingers.

16.2 Apoptosis

Apoptosis is referred as a programmed cell death in which the cell's own molecules are ultimately responsible for its death. This type of cell death is different from the other mode of cell death i.e., necrosis. Morphological changes and DNA fragmentation are the two criteria to distinguish apoptosis from necrosis. The changes that occur in a cell during its apoptosis include –

- Cell shrinkage
- Loss of normal cell-to-cell contact
- Blebbing at the cell surface
- Intense cytoplasmic vacuolization
- Conservation of organelle structure
- Condensation of the chromatin
- Loss of normal nuclear architecture
- Fragmentation of DNA

The changes that occur during necrosis mode of cell death include –

- Critical damage of organelles
- Rupturing of plasma membrane
- Dispersion of cytoplasmic elements into the extracellular space

- Conservation of shape of the nucleus
- Flocculation of chromatin

DNA fragmentation is a biochemical characteristic and the apoptotic cells break up their DNA into multimers of approximately 200 bp. This fragmentation of DNA can be quantified and used as a marker for programmed cell death. Contrastingly, during necrosis the DNA of the dying cell remains intact initially and eventually degraded. Another important characteristic feature of apoptotic cells is the expression of specific markers on their surfaces such as vitronectin and phosphatidylserine which can be recognized by professional phagocytes. These apoptotic cells are rapidly engulfed by the phagocytes before their internal contents are released into the tissue, and therefore the inflammation is prevented. Apoptosis does not stop with the end of embryonic development. In adults, apoptosis leads to the death of cells that no longer required or cells that have sustained irreparable genetic damage and the cells that have the potential to progress into cancer cells. Apoptosis can be divided into two distinct phases –

(1) Commitment phase – when cell responds to signals that commit it to undergoing self destruction.

(2) Execution phase – when death sentence is carried out.

The molecular basis of apoptosis was first revealed from the studies on a nematode worm, *Caenorabditis elegans*. Two features of this worm make it an ideal organism for the study of development and consequently of cell death. First, these worms are transparent, and so the fate of each cell can be microscopically observed in living animals. Second, of 1,090 somatic cells generated during hermaphrodite development, 131 naturally undergo programmed cell death. In mutagenesis studies, two cell death (ced) genes, ced-3 and ced-4, were shown to be required for cell death. If either of these genes was inactivated, death failed to occur and mutant adult worms ended up with ‘extra’ cells. A third gene involved in the regulation of cell death is ced-9. The product of this gene inhibits cell death and functions upstream of ced-3 and ced-4. The discovery of

these genes demonstrated unequivocally that cell death was genetically programmed. Amazingly, the apoptotic program described in *C. elegans* is conserved in mammalian cells since not only are there mammalian homologues of ced-3, ced-4, and ced-9, but also, in some cases, mammalian genes can complement mutations in worms and worm genes are functional in mammalian cells.

In mammals, the program for cell death is more complex than that in works and the induction of apoptosis is highly regulated. Furthermore, there are many homologues of certain cell death genes, suggesting both redundancy and multiple pathways of apoptosis.

16.3 Triggering of Apoptosis

16.3.1 Signal Transduction of a cell Death Signal

Many apoptotic signals are received by the cell through surface receptors. Several mechanisms exist to transduce either the pro- or the antiapoptotic signal from the cell surface to the cell death machinery (Fig. 16.1). The initial signal frequently comes from the binding of a ligand to a specific receptor at the cell membrane. This signal is transduced within the cell and can initiate several pathways, including generation of second messengers like an increase in intracellular cyclic AMP (cAMP) or calcium concentrations, activation of specific kinases that start the apoptotic program, and interaction with molecular adapters that directly connect with cell death effectors. The Fas ligand-induced apoptosis is the simple example of this process.

Fig. 16.1 Models for the induction of Apoptosis

Fas ligand is a molecule related to the cytokine tumor necrosis factor alpha (TNF-α) and is important in T-lymphocyte cytotoxicity. Fas lignad binds to Fas, its receptor, on the target cell. This binding leads to an active receptor complex which transduces a death signal via the cytosolic adapter molecule 'Fas-associating protein with death domain' (FADD). FADD connects the receptor and an effector protease of the caspase family. The ligand-receptor interaction triggers the activation of a protease, which leads to the release of cytochrome C from mitochondria, which in turn activates a series of proteases, whose actions culminate in the destruction of cell structures.

The Fas receptor-bound FADD binds the protein caspase-8 which has a death domain as well as proteolytic activity and may trigger common pathway. Members of the caspase

family (cysteine aspartate proteases) are important downstream components of the pathway. Caspase-8 cleaves a protein called 'Bid' releasing the C-terminal domain which then translocates to the mitochondrial membrane. This causes the release of cytochrome C. This released cytochrome C triggers the interaction of the cytosolic protein Apaf-1 with caspase-9. When Apaf-1 oligomerizes with procaspase-9, this causes the autocleavage that activates caspase-9. This activated caspase-9 in turn cleaves procaspase-3 to generate activated caspase-3. This caspase-3 is homologue to Ced-3 protein of *C. elegans*. The release of cytochrome C from the mitochondria is a crucial control point in the pathway. Bid is a member of the important Bcl-2 family. Caspase-3 acts at the effector stage of the pathway. The pathway that leads to DNA fragmentation has been identified. The caspase-3 cleaves one subunit of a dimmer called 'DNA fragmentation factor' (DFF). As a result the other subunit activates a 'nuclease' that eventually degrades DNA.

16.4 Effector Molecules of Apoptosis

16.4.1 Caspases

Caspases are a family of cysteine proteases that play a central role in the apoptotic pathway. Among cysteine proteases, caspases are unique in requiring an aspartate at the cleavage site. The first caspase to be isolated, interleukin-1β (IL-1β)-converting enzyme (ICE; caspase 1), was identified by classical biochemical studies, using the limited proteolysis of IL- 1β as an assay. IL- β is a cytokine, i.e., a protein that signals to other cells, with significant proinflammatory activity. IL- β is synthesized as a biologically inactive 30-kDa protein which is cleaved to a mature form of 17 kDa. The initial link between ICE and apoptosis was made by sequence comparison; the ICE gene is homologous to the *C. elegans* cell death gene ced-3. Furthermore, both ICE and ced-3 induce apoptosis when over expressed in mammalian cells in tissue culture and ICE complements the ced-3 mutation in *C. elegans*.

More than 10 caspases have been identified in humans. Most of these induce apoptosis when over expressed in tissue culture cells, but not all are required for different apoptosis pathways. Caspase 3 is one of the most commonly activated caspases in apoptosis. The

regulation of caspase activation is one of the key steps in the control of the apoptotic process. The caspase precursors are abundant in the cell cytosol. In response to diverse apoptotic stimuli, they can be proteolytically cleaved into active enzymes. Caspase precursors contain an amino-terminal end that varies in size depending on the caspase and is thought to regulate the cleavage of the precursor. The activation of some caspase precursors is autocatalytic and can lead to cleavage and activation of other members of the family.

The caspase targets appear to be crucial in maintaining the cell architecture, RNA splicing, and DNA repair. These substrates include nuclear lamins, gelsolin, poly(ADP-ribose) polymerase, and the retinoblastoma protein. The hallmark of apoptosis, degradation of DNA into nucleosomal fragments, results from caspase cleavage of a substrate called DNA fragmentation factor. Although caspase activation results in apoptosis, the cascade of programmed cell death that leads from these proteases to apoptosis is not yet understood. It is interesting that although all caspases require an aspartate at the cleavage site, each caspase recognizes a different amino acid sequence, suggesting that they cleave different substrates. The activity of caspase can be blocked by specific inhibitors, like the cellular inhibitors of apoptosis (IAPs). Viruses, like baculovirus and poxvirus, which are strict intracellular pathogens and can benefit from preventing host death encode other caspase inhibitors.

16.4.2 Bcl-2 Family

Several Bcl-2 family members have been identified in mammals and viruses. Members of the bcl-2 family encode proteins that either inhibit (like Bcl-2 and Bcl-xL) or activate (like Bax, Bad, Bik, and Bak) apoptosis. Bcl-2 is homologous to the *C. elegans* apoptosis inhibitor gene ced-9. bcl-2 complements the ced-9 mutation in works and inhibits apoptosis in many different instances when over expressed in mammalian cells. The highest homology between members of this large family is found in two specific regions called Bcl-2 homology domains 1 and 2. Both these domains are crucial for binding to other Bcl-2 homologues. The Bcl-2 family of proteins form homo- and heterodimers which antagonize or enhance the functions of the two partners. For example, over

expression of Bad, which results in apoptosis, leads to both formation of Bad-Bad homodimers and disruption of Bcl-2 – Bcl-2 homodimers to form Bad – Bcl-2 heterodimers. And it appears that the fate of the cell depends on the relative amounts of Bcl-2 inhibitors and activators present.

The Bcl-xL, in addition to its function of binding to Bcl-2 family members, also binds to Ced-4, the product of a gene important in the induction of apoptosis in C. elegans. Ced-4 can bind to and activate caspases. Bcl-2 also prevents cytochrome c release from mitochondria. Cytochrome c is a mitochondrial protein localized in the intermembrane space and is involved in cellular respiration. Intact cells undergo apoptosis after release of cytochrome c in the cytosol, indicating that this protein, in addition to its function in respiration, has a function in apoptosis. Thus, it has been postulated that Bcl-2 could act in situ on mitochondria by inhibiting cytochrome c release.

The mechanism of action of this family of proteins remains elusive. Bcl-2 localizes to the outer mitochondrial membrane and the endoplasmic reticulum and might regulate the redox potential of the cell. Interestingly, the three-dimensional structure of Bcl-xL has revealed striking similarity to the pore-forming subunits of bacterial proteins, such as diphtheria toxin and colicins. The structural similarity led to experiments that indicate that Bcl-xL forms channels in lipid membranes. This activity allows for alterations in mitochondrial permeability by the Bcl-2 family of proteins.

16.5 Induction of Apoptosis by Microorganisms

Many bacterial pathogens that include *Bordetella pertussis, Clostridium difficile, Corynebacterium diphtheriae, Escherichia coli, Helicobacter pylori, Legionella pneumophila, Listeria monocytogenes, Mycobacterium* spp*., Pseudomonas aeruginosa, Salmonella* spp*., shigella* spp*., Staphylococcus aureus, Streptococcus pyogenes* and *Yersinia* spp., induce apoptosis in host cells. These microbial pathogens may be grouped by mechanisms they use to induce apoptosis. The proapoptotic strategies used include activation of cell surface receptors, mimicry of second messengers, regulation of caspase

function, inhibition of protein synthesis, disruption of the host cell membrane, and finally, unknown mechanisms.

16.5.1 Activation of Host Cell Receptors that Signal for Apoptosis

Staphylococcus aureus is a gram-positive coccus that can be part of the normal flora of the skin and mucosa. This microorganism is also the etiological agent of a variety of diseases including skin lesions, food poisoning, toxic shock syndrome, endocarditis, and osteomyelitis. Many virulence factors implicated in staphylococcal virulence, including superantigens, can cause apoptosis. Superantigens are proteins that activate T cells by directly binding both to major histocompatibility complex class Ii molecules on antigen-presenting cells and to the T-cell receptor (TCR) on T cells. *Streptococcus pyogenes* is another gram-positive coccus and is an important cause of pharyngitis and is sometimes associated with serious sequelae. It has also been associated with toxic shock-like syndrome through exotoxins that act as superantigens.

The superantigens of streptococci and staphylococci recognize specific TCRs. Engaging the TCR on T cells can activate programmed cell death. Activation of the TCR through superantigens like staphylococcus exotoxin B (SEB), SEA, SED, and SEE induce apoptosis in T cells and thymocytes both in vitro and in vivo. Although that superantigens activate apoptosis by engaging the TCR, the precise pathway by which superantigen activation results in programmed cell death is not yet known. However, it is clear that T cells have to be active and proliferating in order to die. SEB-induced cell death is mediated by both protein kinase C and an ATP-gated ion channel.

16.5.2 Induction of Second Messengers

Bordetella pertussis, a gram-negative rod, is the etiological agent of whooping cough, an upper respiratory tract infection characterized by a cough with an inspiratory “whoop”. *Bordetella* is high contagious and is transmitted through air droplets. In the course of the infection, the bacteria remain localized to the respiratory tract, where they evoke an acute inflammation. *B. pertussis* kills macrophages by apoptosis in vitro by secreting adenylate cyclase-hemolysin (AcHly) toxin. This toxin has two domains: (i) a potent adenylate

cyclase activity, which is activated by calmodulin, and (ii) a hemolysin activity, which is a pore-forming protein that is thought to allow the translocation of the cyclase into the host cell cytoplasm. AcHly kills macrophages only when both parts of the toxin are functional. This indicates that the disruption of the host cell cytoplasmic membrane through the pore-forming domain of this toxin is not sufficient to kill the cell. An increase in the intracellular concentration of camp triggers pathways that lead to apoptosis, and curtailing the production of this second messenger can prevent programmed cell death. Hence, it is interesting to speculate that *Bordetella* initiates apoptosis by sharply increasing the intracellular concentrations of cAMP to activate a program for cell death.

Bordetella encodes another toxin, pertussis toxin (PT), which also increases the intracellular camp concentration. PT is a member of the A/B family of toxins, in which the B subunits allow the translocation of the enzymatically active A subunit into the cytoplasm of the target cell. PT ADP-ribosylates a G protein, inhibiting the inhibitory subunit which acts on the host adenylate cyclase. Thus, indirectly, by inhibiting the down-regulator of the host cell endogenous cyclase, PT activity leads to an increase in the intracellular cAMP concentration. Interestingly, PT is not necessary for *Bordetella*-mediated induction of macrophage cytotoxicity. The importance of *Bordetella*-induced apoptosis is still not understood. AcHly is produced early in the infection and is then down-regulated during the later, chronic phase of the disease. Therefore, it is possible that *Bordetella* kills alveolar macrophages to eliminate the first line of defense that it encounters. Alternatively, early macrophage apoptosis might be important in triggering an inflammatory response.

16.5.3 Activation of Caspases

Shigella is a gram-negative rod that causes dysentery, a severe form of diarrhea that often contains blood and mucus. *Shigella* is transmitted through the fecal-oral route and is an extremely infectious agent. *Shigella* is an invasive organism that induces macrophage apoptosis. This pathogen is phagocytosed by macrophages but escapes from the phagocytic vacuole. Inside the cytoplasm of the macrophage, S*higella* secretes, among other proteins, invasion plasmid antigen B (IpaB). IpaB disseminates throughout the

cytoplasm, binding to and activating ICE (Caspase 1). The activity of mature ICE is responsible for both macrophage apoptosis and maturation of IL-1β. *Shigella* induces classical apoptosis, as determined by the morphological changes and the fragmentation of the host cell DNA. Since IpaB has to be delivered by the bacterium into the compartment where ICE resides, *Shigella* is able to induce apoptosis only from within the cytoplasm. All clinical isolates of *Shigella* tested so far induce macrophage cell death. Apoptosis is up-regulated in tissues of animals experimentally infected with *Shigella* and in patients suffering from shigellosis. The findings strongly suggest that macrophage apoptosis is an important step in *Shigella* pathogenesis.

Salmonella, like Shigella, is a gram-negative enteric pathogen that is transmitted orally. Depending on the bacterial serovar and host specificity, Salmonella either remains localized in the gust and produces gastroenteritis or is dispersed hematogenously to other organs such as the spleen and the liver, as in typhoid fever. Salmonella can also kill macrophages by apoptosis, although the mechanism is unclear. It has been demonstrated that during Salmonella-induced apoptosis there is no autocrine induction of a death signal by macrophages. It also appears that Salmonella, like Shigella, induces apoptosis only when it is inside macrophages. Only bacteria competent for invasion and capable of generating characteristic ruffles in the host cell membrane kill macrophages, whereas the non-invasive mutants do not activate programmed cell death. Salmonella, unlike Shigella, induces a significant increase in the concentration of intracellular calcium when it invades host cells. This increase in the intracellular calcium concentrations can act as a second messenger to activate apoptosis.

16.5.4 Inhibition of Protein Synthesis

Corynebacterium diphtheriae, a non-spore forming gram-positive rod, and the gram-negative bacteria *Pseudomonas aeruginose, Shigella dysenteriae*, and enterohemorrhagic *Escherichia coli* (EHEC) secrete toxins that inhibit eukaryotic translation and activate apoptosis in host cells. All these toxins are of the A / B type, like PT. They inhibit translation through different mechanisms. Inhibition of eukaryotic translation or

transcription by other substances, like cycloheximide or actinomycin, also eventually activates apoptosis in many cell types.

C. diphtheriae lives extracellularly and secretes diphtheria toxin (DT). The B subunit of DT binds to an extracellular glycoprotein receptor, allowing the toxin to be endocytosed by several different cell types including epithelial and myeloid lines. The acidic environment of the phagolysosome exposes a specific domain and creates an aqueous pore that allow the translocation of the A subunit into the host cell cytoplasm. The A subunit is an enzyme that ADP-ribosylates elongation factor 2 (EF-2), an essential component of the translation machinery. The ribosylation inhibits EF-2 activity and blocks protein synthesis, eventually leading to apoptosis. Three lines of evidence strongly support the hypothesis that DT induces apoptosis by inhibiting translation: (i) the levels of inhibition of protein synthesis and cytotoxicity correlate very tightly in DTX-treated cells; (ii) host cells carrying mutations in EF-2 which prevent ADP ribosylation are insensitive to DTX-induced apoptosis; and (iii) inhibition of ADP-ribosylation by DT blocks both cytotoxicity and the inhibition of protein synthesis.

Pseudomonas aeruginosa is found in soil and water and sometimes in the flora of the gut. It can cause sepsis, urinary tract infection, and pneumonia, particularly in immunocompromised persons and cystic fibrosis patients. One of the key virulence factors of *P. aeruginosa* is exotoxin A (ExoA), which, like DT, ADP-ribosylates EF-2 and inhibits protein synthesis. ExoA and DT do not have significant sequence homology. However, ExoA also induces apoptosis in a human monoblastoid cell line. Mutations in EF-2 confer resistance to ExoA, suggesting that, as with DT, inhibition of translation is necessary for ExoA-mediated apoptosis.

Shigella dysenteriae and EHEC cause a dysenteric syndrome and can be associated with sequelae involving the kidneys and the central nervous system. *S. dysenteriae* and EHEC both produce toxins, Shiga toxin (ST) or Shiga-like toxins (SLT), respectively, which are almost identical. The B subunits of these A/B toxins mediate binding to globotriaosylceramide (Gb_3), the receptor on the host cell membrane. The A subunit

cleaves eukaryotic rRNA and disrupts ribosomal function and hence inhibits protein synthesis. Induction of apoptosis by these toxins has been documented both in vitro and in vivo. The purified B subunit of the toxin is sufficient to kill epithelial cells, suggesting that Gb_3 binding might activate a signal transduction cascade that culminates in apoptosis.

16.5.5 Disruption of the Cytoplasmic Membrane

Several pore-forming proteins (PFP) are made by microbes. These toxins include *Staphylococcus aureus* alpha-toxin, *Actinobacillus actinomycetemcomitans* leukotoxin, *Listeria monocytogenes* listeriolysin, and *E. coli* hemolysin. It appears that large doses of PFP massively disrupt the integrity of the cell membrane and cause necrosis. At lower doses, a more delicate modification of the host cell membrane results in apoptosis. It is still unclear how disruption of the cell membrane can lead to apoptosis. Nevertheless, mutations in PFP that lower their hemolytic activity also decrease their apoptotic potential, demonstrating that the pore-forming ability is directly linked to apoptosis.

A. actinomycetemcomitans is a gram-negative coccobacillus that is associated with periodontitis as well as meningitis and endocarditis. It produces leukotoxin which causes cytotoxicity in lymphoid cells but spares fibroblasts, platelets, and endothelial and epithelial cells. In addition, *Actinobacillus* induces apoptosis independently of leukotoxin. There are *Actinobacillus* strains that do not make leukotoxin but still kill macrophages. This second pathway requires intracellular bacteria and involves a protein kinase C pathway but not a camp-dependent protein kinase pathway.

Listeria monocytogenes is a gram-positive rod that can cause meningitis and sepsis in infants and immunosuppressed patients. It is transmitted orally, and after invading the gastrointestinal tract, it can spread systemically. *Listeria* invades cells and escapes from the phagolysosome into the cytoplasm of the host cell. It can kill cells, however, without invading them. *Listeria* induces apoptosis in dendritic cells and hepatocytes, but it is not cytotoxic to macrophages. Dendritic cells are antigen-presenting cells located in lymphoid aggregates throughout the body including the gut. Killing of dendritic cells

might be an important way of preventing the immune system from mounting a timely immune response against this pathogen. When *Listeria* infects the liver, it induces hepatocyte apoptosis.

16.6 Summary

Apoptosis is otherwise known as programmed cell death, is a natural process by which the host controls cell numbers and it is also important in embryogenesis and the response to genetic damage. In contrast to necrotic cell death, apoptosis is a highly regulated process which involves the active participation of the cell and is controlled by complex signal transduction pathways. Apoptosis can be induced by a wide variety of factors, including radiation, hyperthermia, withdrawl of growth factors, glucocorticoids, certain cytokines and various pharmacological agents such as inhibitors of kinases. However, the common pattern of morphological and biochemical changes in cells undergoing apoptosis suggested that there was a single pathway of mortality which was independent of the apoptotic stimulus. This pathway involves a group of related enzymes, which are called caspases, that are present in the cell as inactive pro-forms which can be activated by a variety of stimuli. These proteases, either as part of a cascade or as a collection of overlapping enzymes, cleave key cellular proteins and result in cells dying without releasing components which could cause further tissue damage. Some bacteria have evolved means of inducing this process. For example, invasion of macrophages by *Salmonella typhimurium* has been shown to induce apoptosis.

Much of the understanding of apoptosis came from the study of the nematode *Caenorhabditis elegans*. In this organism, it was demonstrated that mutation of the gene ced-3, which encodes for one of the caspases, blocks apoptosis. Mutation studies showed that another gene in this worm, ced-9, was responsible for inhibiting apoptosis. In vertebrates the counterpart of ced-9 is known as bcl-2. Bcl-2 belongs to a family of proteins that can undergo homo- or hetero-dimerization. One of these proteins is termed Bax and counteracts the ability of Bcl-2 to protect against apoptosis. It is likely that this Bcl-2 / Bax interplay is a key controlling element in apoptosis.

16.7 Model Questions

1. Define Apoptosis? Write an account on the effector molecules of apoptosis.
2. Give an account on the role of bacteria in the induction of apoptosis.
3. Short questions
   (i) Caspases
   (ii) Apoptosis vs Necrosis

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