PAPER V

IMMUNOLOGY AND ANIMAL BIOTECHNOLOGY

II B.Sc. SEMESTER IV

SUBJECT – ZOOLOGY

Paper –IMMUNOLOGY &ANIMAL BIOTECHNOLOGY

UNIT – I

1.INNATE IMMUNITY

The immune system, comprising cells, the molecules they produce, and the organs that organize those components, evolved over millions of years in response to infections with pathogenic microorganisms. Its essential role in maintaining health is based on its recognition and elimination or control of those foreign microbes. Central to the success of the protective role of the immune system is its capacity to distinguish foreign and dangerous invaders from self-components.

The immune system is generally described as including an *innate* immune system and an*adaptive* immune system. The former provides the first and rapid line of defence and cellular response to a foreign stimulus. The latter, dependent on activation by the innate immune response, develops a more specific response targeted to the offending organism and generates memory for that stimulus that can be elicited rapidly should that organism be encountered again on a later occasion

Definition of Innate Immunity -

The innate immune system is the first part of the body to detect invaders such as viruses, bacteria, parasites and toxins, or to sense wounds or trauma.

Upon detection of these agents or events, the innate immune system activates cells to attack and destroy the outsider, or to initiate repair, while also informing and modulating the *adaptive* immune response that follows this first line of defence.

Innate Immunity operates through many factors such as

A. Physical and Mechanical

B. Biochemical Factors

C. Cellular factors

D. Genetic Factors

E. Body Temperature

F. Inflammation

G. Fever

A. Physical And Mechanical Factors –

1.**Skin-** In vertebrates, the skin and other epithelial surfaces, including those lining the lung and gut , provide a physical barrier between the inside of the body and the outside world. Tight junctions between neighboring cells prevent easy entry by potential pathogens.

2. Mucous Membrane and Cilia - The interior epithelial surfaces are also covered with a mucus layer that protects these surfaces against microbial, mechanical, and chemical particles. The slimy mucus coating is made primarily of secreted mucin and other glycoproteins, and it physically helps prevent pathogens from adhering to the epithelium. It also facilitates their clearance by beating cilia on the epithelial cells.

3. Coughing and Sneezing- The mechanical action such as cough and sneeze also drive out the foreign particles that enter the digestive and respiratory passage.

4. **Tears, saliva and urine-** tears continually released by lacrimal ductscontain antimicrobial substances (e.g. lysozyme), wash away any

microbes, debris on the eye surface.The saliva in mouth wash out if any microbe enters the mouth . And the urine sendout the microbesfrom urethra.

B. Biochemical factors-

1. Secretions of Skin- The high concentration of salt has bactericidal property. The secretions of sweat and sebaceous glands have bactericidal and fungicidal property.

2. Secretions of Digestive tract- acidic environment of stomach due to release oh Hcl kills most microbes.

3. Human milk- Human milk is rich in antibacterial substances namely lactoferritin and viruses.

4. Lysozyme- Lysozyme is a naturally occurring enzyme found in bodily secretions such as tears, saliva, and milk. It functions as an antimicrobial agent by cleaving the peptidoglycan component of bacterial cell walls, which leads to cell death.

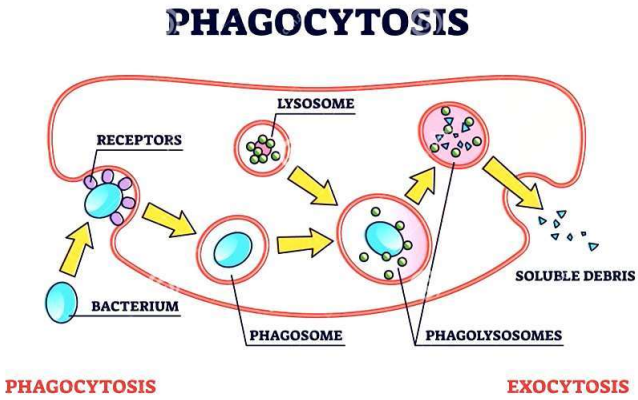
5. Interferons- These are a group of soluble ,non-toxic ,glycoproteins produced in small amounts by all cells of the body. This is an anti-viral agent which inhibits intracellular viral replication in cells infected with virus.

c. Cellular factors-

1. **Phagocytosis-** Phagocytosis is a process by which certain living cells called phagocytes ingest or engulf other cells or particles. The phagocyte may be a free-living one-celled organism, such as an amoeba, or one of the body cells, such as a white blood cell. In higher animals phagocytosis is chiefly a defensive reaction against infection and invasion of the body by foreign substances.

Process-

The process of phagocytosis involves the following stages- Chemotaxis, Attachment, Ingestion, Intracellular killing and Digestion.



2. Natural killer cells- Natural Killer (NK) Cells are lymphocytes in the same family as T and B cells, coming from a common progenitor. However, as cells of the innate immune system, NK cells are classified as group I Innate Lymphocytes (ILCs) and respond quickly to a wide variety of pathological challenges. NK cells are best known for killing virally infected cells, and detecting and controlling early signs of cancer. As well as protecting against disease, specialized NK cells are also found in the placenta and may play an important role in pregnancy.

NK cells were first noticed for their ability to kill tumour cells without any priming or prior activation (in contrast to cytotoxic T cells, which need priming by antigen presenting cells). They are named for this 'natural' killing. Additionally, NK cells secrete cytokines such as IFNγ and TNFα, which act on other immune cells like Macrophage and Dendritic cells to enhance the immune response.

Functions

(1) NK cells are cytotoxic for tumor cells and virally infected autologus cells.

(2) They also have been reported to play a role in resistance to some bacterial, fungal, and parasitic infections, and to participate in regulation of the immune response through the secretion of lymphokines such as IL-2.

(3) Recent evidence suggests that NK cells, not killer (K) cells, may be responsible for antibody-dependent cell-mediated cytotoxicity (ADCD).

D.Genetic factors-

1**. Species Immunity-** When a disease attacks a species only and another species is entirely immune to it, it is known as species immunity. For instance, only human beings are susceptible to ailments such as measles, mumps, HIV, etc. Other animals like dog cannot contract these diseases.

2. **Racial Immunity-** When one race is immune to a certain disease, and another race is susceptible to it, it is referred to as racial immunity. Again, factors such as genetic make-up, food habits, climate conditions play an essential part in determining racial immunity.

3. **Individual Immunity-** Sometimes, individuals belonging to the same race and who have been exposed equally to the virus, pathogens or worms can show a different level of immunity to a disease or infection. This can be due to any number of reasons. People who are exhibiting higher levels of immunity may have been exposed to the virus before, and their body has developed resistance against it. Other factors such as health, age and hereditary traits can also be responsible for individual immunity.

E. Body temperature – Temperature is also important to determine the innate immunity. For example- the Tubercle bacilli which are pathogenic to mammals will not infect the cold blooded animals. Hens are naturally immune to Anthrax But they can be infected ,if the body temperature is lowered.

F. Inflammation- Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants, and is a protective response involving immune cells, blood vessels, and molecular mediators. The function of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and initiate tissue repair.

G. Fever- A raise in body temperature following infection is a natural defense mechanism. Raise in temperature may kill the harmful pathogens.

II. ACQUIRED IMMUNITY

The immunity that an individual acquires after the birth is called acquired or adaptive or specificimmunity. It is specific and mediated by antibodies or lymphocytes or both which make the antigen harmless.It not only relieves the victim of the infectious disease but also prevents its further attack in future.The memory cells formed by B cells and T cells are the basis of acquired immunity. Thus acquiredimmunity consists of specialized B and T lymphocytes and Antibodies.

Characteristics of Acquired Immunity:

Specificity: It is the ability to differentiate between various foreign molecules (foreign antigens).

Diversity: It can recognise a vast variety of foreign molecules (foreign antigens).

Discrimination between Self and Non-self:
It can recognise and respond to foreign molecules (non-self) and can avoid response to those molecules that are present within the body (self) of the animal.

Memory: When the immune system encounters a specific foreign agent, (e.g., a microbe) for the first time, it generates immune response and eliminates the invader. This is called first encounter. The immune system retains the memory of the first encounter. As a result, a second encounter occurs more quickly and abundantly than the first encounter.

Types of Acquired Immunity:

Acquired (= Adaptive) Immunity is of two types: active immunity and passive immunity.

1. Active Immunity:
In this immunity person's own cells produce antibodies in response to infection or vaccination. It is slow and takes time in the formation of antibodies. It is long lasting and is harmless. Active immunity may be natural or artificial.

(a) **Natural Active Immunity**- A person who has recovered from an attack of small pox or measles or mumps develops natural active immunity.

(b) **Artificial active immunity –** It is the resistance induced by vaccines. Examples of vaccines are asfollows: Bacterial vaccines, (a) Live- BCG vaccine for tuberculosis, (b) Killed vaccines- TAB vaccine forenteric fever. Viral vaccines, (a) Live – sabin vaccine for poliomyelitis, MMR vaccine for measles,mumps, rubella, (b) Killed vaccines- salk vaccine for poliomyelitis, neural and non-neural vaccines forrabies. Bacterial products. Toxoids for Diphtheria and Tetanus.

2. Passive Immunity:
When ready-made antibodies are directly injected into a person to protect the body against foreign agents, it is called passive immunity. It provides immediate relief. It is not long lasting. It may create problems. Passive immunity may be natural or artificial.

(a) **Natural passive immunity –** It is the resistance passively transferred from the mother to the foetus through placenta. IgG antibodies can cross placental barrier to reach the foetus.
After birth,immunoglobulin's are passed to the new-born through the breast milk. Human colostrum (mother'sfirst milk) is rich in IgA antibodies. Mother's milk contains antibodies which protect the infant properly by the age of three months.

(b) **Artificial passive immunity**- It is the resistance passively transferred to a recipient by administration of antibodies. This is done by administration of hyper-immune sera of man or animals. Serum (pi.sera) contains antibodies. For example, anti-tetanus serum (ATS) is prepared in horses by active immunisation of horses with tetanus toxoid, bleeding them and separating the serum. ATS is used for passive immunisation against tetanus. Similarly anti-

diphtheric serum (ADS) and anti-gasgangrene serum (AGS) are also prepared.

- Hyperimmune serum of animal or Human origin
- Convalescent Serum
- Polled sera of healthy individuals

III. DIFFERENT TYPES OF CELLS IN IMMUNE SYSTEM

The cells concerned with the defense of the body are called immune cells. Immune cells are the army of the defense system. They are the soliders of the immune system. They function as sentries, spies, wrestlers and producers of weapons and toxins.

The following are the important immune cells.

1. Lymphocytes
2. Phagocytic cells
3. Granulocytic cells
4. Antigen presenting cells

1.Lymphocytes

As stated above, lymphocytes are the primary cells of adaptive immune responses .The two basic types of lymphocytes, B cells and T cells, are identical morphologically with a large central nucleus surrounded by a thin layer of cytoplasm. They are distinguished from each other by their surface protein markers as well as by the molecules they secrete. While B cells mature in red bone marrow and T cells mature in the thymus, they both initially develop from bone marrow. T cells migrate from bone marrow to the thymus gland where they further mature. B cells and T cells are found in many parts of the body, circulating in the bloodstream and lymph, and residing in secondary lymphoid organs, including the spleen and lymph nodes, which will be described later in this section. The human body contains approximately 1012 lymphocytes.

B Cells

B cellsare immune cells that function primarily by producing antibodies. An antibodyis any of the
group of proteins that binds specifically to pathogen-associated moleculesknown as antigens.An antigenis a chemical structure on the surface of a pathogen that binds to T or B lymphocyteantigen receptors. Once activated by binding to antigen, B cells differentiate into cells that secrete asoluble form of their surface antibodies. These activated B cells are known as plasma cells.

T Cells

The **T cell**, on the other hand, does not secrete antibody but performs a varietyof functions in theadaptive immune response. Different T cell types have the ability to either secrete soluble factorsthat communicate with other cells of the adaptive immune response or destroy cells infected withintracellular pathogens. The roles of T and B lymphocytes in the adaptiveimmune response will be discussed further in this chapter.

Plasma Cells

Another type of lymphocyte of importance is the plasma cell. A plasma cellis a B cell that hasdifferentiated in response to antigen binding, and has thereby gained the ability to secrete solubleantibodies. These cells differ in morphology from standard B and T cells in thatthey contain a largeamount of cytoplasm packed with the protein-synthesizing machinery known as rough endoplasmicreticulum.

Natural Killer Cells

A fourth important lymphocyte is the natural killer cell, a participant in the innate immune response.

A natural killer cell (NK)is a circulating blood cell that contains cytotoxic (cell-killing) granules in itsextensive cytoplasm. It shares this mechanism with the cytotoxic T cells of the adaptive immuneresponse. NK cells are among the body's first lines of defense against viruses and certain types ofcancer.

2.Phagocytic cells:

Monocytes and macrophages are mononuclear phagocytic cells.

Granulocyte-monocyte progenitor cell differentiates into promonocytes and neutrophil.Promonocytes leaves the bone marrow and enter into blood stream where theydifferentiate into mature monocytes.Monocytes circulates

in blood for about 8 hours, during which they enlargesand then enterinto tissues and differentiates into specific macrophages and dendritic cells.

1. Monocytes:

Blood monocytes measure 12-15 μm with a single lobed kidney shaped nucleus.It accounts for (2-8%) of blood leucocytes.

Immunological Functions of monocytes:

- Helps in antigen processing and presentation
- Releases cytokines
- Specialized function in tissues
- Cytotoxicity

2. Macrophages:

Monocyte migrates to tissue and differentiates into macrophages.Differentiation of monocytes into macrophages involves following changes:

Cell enlarges 5-10 foldsIntracellular granules increases in number and complexityIncrease phagocytic abilityProduces higher level of hydrolytic enzymes and cytokine.

Macrophages serve different functions in different tissues.Alveolar macrophages : in lungs,Histiocyte: connective tissue,Kuffer cell: liver ,Microglial cell: brainOsteoclast: bone

Immunological functions of macrophages:

- Phagocytosis
- Antigen presentation to T-cell
- Secretion of lymphokines IL-1, IL-6. IL-12. TNF-α etc to activates inflammatory response
- Secretion of granulocyte monocyte colony (GMC) stimulating factors.

3. Granulocytic cells:

a. Neutrophil:

Neutrophils are (11-14μm) in diameter with multilobed nucleus with granules in cytoplasm.It constitutes 50-70 % of total circulating WBC and remains for 7-8 hours in blood and thenmigrates to tissues.Life span is 3-4 days.Also known as polymorphonuclear (PMN) leucocyte.

Neutrophils is stained by both acidic and basic dye.

Immunological functions of Neutrophil:

- Phagocytic role in acute inflammatory response.
- It is the first immune cell to responds in inflammation.

b.Eosinophils:

Eosinophils are (11-15μm) in diameter, heavily granulated with bilobed nucleus
It is stained by acidic dye ie Eosin
They are phagocytic and motile
Immunological functions of Eosinophil:

- Granules contain various hydrolytic enzymes that kill parasites which are too large to be
- phagocytosed by neutrophils.
- Provide allergic inflammation

c.Basophils:

Basophils are non-phagocytic cell found in small number in blood and tissue
Cytoplasm contains large number of prominent basophilic granules containing histamine,heparin, serotonin, and other hydrolytic enzymes.Stained by basic dyes.
Immunological functions:

- Provide anaphylactic and atopic allergic reaction

4. Antigen presenting cells

An antigen-presenting cell (APC) or accessory cell is a cell that displays antigen bound by major histocompatibility complex (MHC) proteins on its surface; this process is known as antigen presentation. T cells may recognize these complexes using their T cell receptors (TCRs). APCs process antigens and present them to T-cells. They are Dendritic cells, Macrophages and B cells.

Dendritic cell

Dendritic cells have long cytoplasmic externsions that resembles to dendrites of nerve cell.They have highly pleomorphic with a small central body and many long needle likeprocesses.Dendritic cells are antigen presenting cell (APC) because they possess MHCclass.

Immunological functions:

- Involved in antigen presentation to T-cells during primary immune response.
- Very little role in phagocytosis.

IV. DIFFERENT TYPES OF LYMHOCYTES

Lymphocytes are a type of white blood cells present in the blood and lymph of our body. They are responsible for adaptive or acquired immunity. They are a type of agranulocytes. Around 20-25% of white blood cells are B and T lymphocytes. 99% of the cells of lymph are lymphocytes.

There are three types of lymphocytes, i.e. B-lymphocytes, T-lymphocytes and Natural killer cells (NK cells). They differ in their structure and function. There are surface proteins present, which differentiate the different subtypes of lymphocytes. They are known as the cluster of differentiation or CD markers.

Lymphocytes are concentrated in the lymphoid organs, e.g. spleen, lymph nodes, tonsils, etc. and initiate the immune response against the foreign pathogen.

All the lymphocytes are produced from the stem cell in the bone marrow and later mature and differentiate in the specific organs. B- lymphocytes mature in the bone marrow, whereas T-lymphocytes mature in the thymus. B and T lymphocytes later differentiate into effector and memory cells on exposure to antigens.

Lymphocytes are responsible for both humoral (antibody-mediated) and the cell-mediated immune (CMI) response by B and T lymphocytes, respectively.

Lymphocytes Types

On the basis of structure and function, lymphocytes are divided into three main types:

- B Cells
- T Cells
- Natural Killer (NK) Cells

B Lymphocytes

B cells get their name from the site of maturation in the birds, where they were first discovered, i.e. *bursa* of Fabricius. In humans and some other mammals, the main site of B lymphocytes maturation is the bone marrow.

The mature B cell synthesizes and expresses the specific antibodies produced in response to the antigen. It binds to the specific antigen by the membrane-bound immunoglobulin or antibody, which is also known as BCR or B-cell receptor.

The activated B cells further differentiate into plasma cells or effector cells. They lose the surface antibody and start producing the specific antibodies in a large amount to fight the infection.B lymphocytes produce antibodies, hence they are known to trigger the humoral immune response.Memory B cells are formed after primary infection and they remain in the blood for decades. They circulate in the blood, identify and act against previously infected antigens.

T Lymphocytes

T cells also get their name from the site of maturation, i.e. thymus. T cells also have surface receptors to recognize antigens but they do not directly bind to the antigens like surface antibody receptors on B cells.T cell receptors recognize antigens bound to a special kind of cell membrane protein known as major histocompatibility complex or MHC molecules.

T lymphocytes differentiate into two main subtypes:

- **T helper (T_H) cells-** They generally contain CD4 membrane glycoprotein on their surface and recognise antigens with class II MHC.The T helper cells trigger various different types of immune cells to act against the antigens like macrophages, B lymphocytes and cytotoxic T cells. The effector T cells secrete different types of cytokines, which directs the immune response by other cell types.
- **T cytotoxic (T_C) cells-** They generally contain CD8 membrane glycoprotein on their surface and recognise antigens with class I MHC.After activation, they proliferate and differentiate into cytotoxic T lymphocytes. It eliminates virus-infected cells, tumour or cancerous cells and also foreign grafts, etc.
- **T Regulatory celis -**Another type of T cells known as the regulatory T cell, regulates the immune response produced.The memory T cells are antigen-specific T cells which have a longer life span. They play a key role in rapid immune response on the re-exposure of an infectious agent. They are responsible for the secondary response.

Lymphocytes Function

- Lymphocytes play a major role in adaptive immunity. The B cells are responsible for humoral immunity and T cells are required for the cell-mediated immunity.
- The B cells secrete antibodies, which are transported by blood hence they can work over a long distance, whereas T cells can migrate to the target tissues and act locally.
- The acquired immune response is specific to the pathogen. Once acquired, it is stored in the memory cell, so that when the infection is repeated, it triggers a highly amplified secondary response.

Natural Killer Cells

- These are large granular lymphocytes appear to function in immune surveillance. a. Source and location
- NK cells are innate, or naturally occurring cytotoxic lymphocytes: they are present in the body from the time of birth, and are not induced by immunologic insult.
- They arise from bone marrow precursors but are of a lineage distinct from that of either T or B cells.
- NK cells make up 10% to 15 % of the lymphocytes in the peripheral blood and 1 % to 2 % of the lymphocytes in the spleen. They are absent from the lymph nodes. 14 b.

Functions

- NK cells are cytotoxic for tumor cells and virally infected autologus cells.
- They also have been reported to play a role in resistance to some bacterial, fungal, and parasitic infections, and to participate in regulation of the immune response through the secretion of lymphokines such as IL-2.
- Recent evidence suggests that NK cells, not killer (K) cells, may be responsible for antibody-dependent cell-mediated cytotoxicity (ADCD).

IV. PRIMARY LYMPHOID ORGANS

The immune system is found throughout the body and is made up ofmany different cells, organs, and tissues.
The organs and tissues of the system can be classified into two maingroups:

(1) Primary lymphoid organs, in which lymphocytes are generatedand undergo development and maturation; and
(2)Secondary lymphoid organs and tissues, where maturelymphocytes interact with antigen.

The vessels of the blood and lymphatic systems connect lymphoid organs and tissues and unite them into a functional whole. Leukocytes, or white blood cells, are found within the blood, lymph and lymphoid tissues and organs. The vertebrate immune system contains many types of leukocytes, but only the lymphocytes have the attributes of receptor diversity, antigen specificity, and self/nonself recognition that are the hallmarks of adaptive immunity.

1. Central lymphoid organs/Primary Lymphoidal Organs

Central lymphoid organs are requisite for the development of the lymphoid system and, therefore, the immune system. These include the thymus, bone marrow, and bursa of Fabricus. Central lymphoid organs are also termed primary lymphoid organs. They are sites where lymphocytes are generated. Both T and B cells originate in the bone marrow but only B cells mature there. Human T cells mature in the thymus.

2. Peripheral lymphoid organs/Secondary Lymphoidal organs

Peripheral lymphoid organs are not required for ontogeny of the immune response. They are sites where adaptive immune responses are initiated and where lymphocytes are maintained. Peripheral lymphoid organs are also termed secondary lymphoid organs. They include the lymph nodes, spleen, tonsils, and mucosal-associated lymphoid tissues in which immune responses are induced.

1. Primary Lymphoidal organs

In the primary lymphoid organ, maturation of lymphocytes takes place and these lymphocytes become committed to a particular antigenic specificity. Only when the lymphocytes mature in the primary lymphoid organ, they become immune competent cells. In mammals B cell maturation occurs in the bone marrow and T cells maturation occurs in the thymus.

A. Thymus :

Thymus is situated above the heart and it is bilobed structure. Thethymus is internally zonated into many lobules, which are separated fromeach other by connective tissue strands called trabeculae. Each lobuleconsists of central medulla and the outer cortex. The medulla is sparselypopulated by thymocytes, whereas the cortex is densely packed withimmature T cells called thymocytes. It is believed that the progenitor T

cells enter the thymus and start proliferating rapidly within the cortex.Both the cortex and medulla of the thymus is composed of network ofstromal cells, epithelial cells, interdigitating dendritic cells and the macrophages. These cells contribute for the maturation of thymocytes.For example, the developing thymocytes and the stromal cells physically interact. The thymic epithelial cells act as nurse cells by having longmembrane processes that surround as many as 50 thymocytes, resultingin the formation of multicellular complexes.

B.Bone Marrow

In mammals B cell maturation occurs in bone marrow, whereas in birdsbursa fabricus is the primary site of B cell maturation. Immature B cells Proliferate and differentiate within the bone marrow. As in the case of Thymus, stromal cells interact directly with B cells and secrete certain cytokines, which help in development of B cells. As observed in thymus,during T cell maturation a selection process occurs with respect to elimination of T cells with self reactive receptors against self-antigens, similarly within the bone marrow elimination of B cells with self reactive antibody receptors occurs.

.There are two categories of bone marrow tissue: Red marrow and Yellow marrow.

a.Red Bone Marrow

Most of the bone marrow during birth to early adolescence is red marrow while the red marrow is replaced with yellow with age. In adults,red marrow is confined mostly to skeletal system bones that serve to produce blood cells and help remove old cells from circulation. They contain hematopoietic stem cells that produce two other types of stem cells myeloid stem cells and lymphoid stem cells. These cells develop into red blood cells, white blood cells, or platelets.

b.Yellow Bone Marrow-

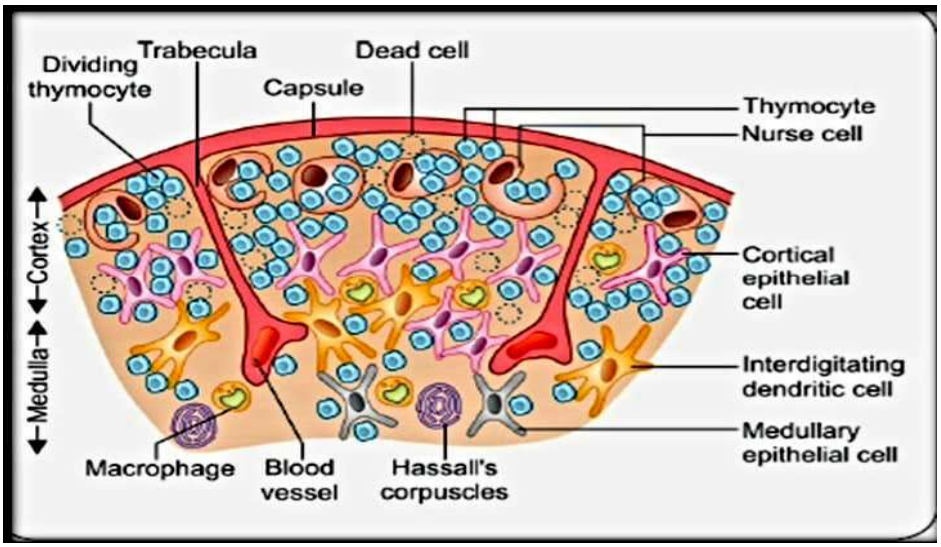
Yellow marrow found in spongy bones and in the shaft of longbones, is non-vascular and consists primarily of fat cells. It is composed of hematopoietic tissue that has become inactive.

C. Bursa of Fabricius

The bursa of Fabricius is a structure peculiar to Aves. It is a blind sac connected by a small duct to the dorsal part of the cloaca. Often nicknamed "the cloacal thymus," the function of the bursa is believed to be similar to that of the thymus . There is no question that the bursa of Fabricius functions as a lymph gland during the first two to three months after the chicken hatches . Like the thymus, the bursa in birds is believed to have some endocrine function in relation to growth and sexual development.

The bursa develops as a dorsal diverticulum of the proctadael region ofthe cloaca. The luminal (interior) surface of the bursa is plicated with as many as 15 primary and 7 secondary plicae or folds. These plicae have hundreds of bursal follicles containing follicle-associated epithelial cells, lymphocytes, macrophages, and plasma cells. Lymphoid stem cells migrate from the fetal liver to the bursa during ontogeny. In the bursa,these stem cells acquire the characteristics of mature immune competent B cells. The bursa is active in young birds. It atrophies after about six months.

T.S of Thymus



VI. SECONDARY LYMPHOID ORGANS

Lymphoid organs are the organs where lymphocyte origin, maturation, and proliferation occurs. There are two types of lymphoid organs they are primary lymphoid organs and secondary lymphoid organs

Primary lymphoid organs

The origin of lymphocytes occurs inside primary lymphoid organs. Those involve the thymus and the bone marrow.

- This is the site where lymphocytes are produced and mature.
- It is also the location where stem cells differentiate and mature into B and T cells
- Humans have two primary lymphatic organs – the thymus gland and the red bone marrow
- B and T cells are formed in the bone marrow
- B cells mature in the bone marrow while T cells mature once they migrate to the thymus.

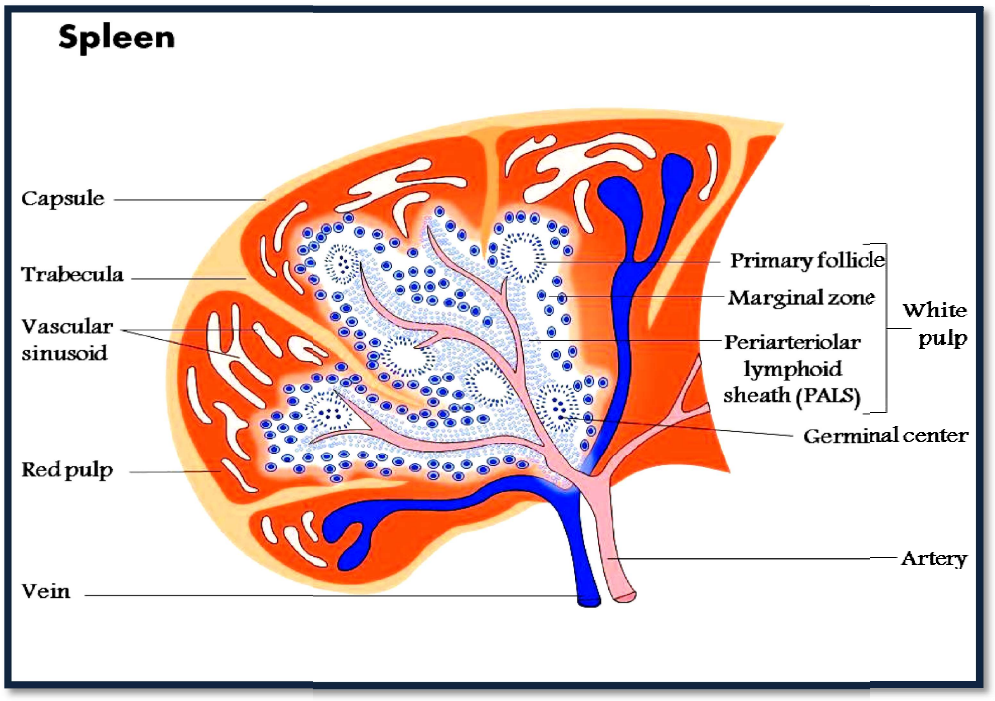
SECONDARY LYMPHOID ORGANS

Upon maturation, the lymphocytes move to a secondary lymphoid organ such as the spleen, lymph nodes, tonsils, small intestine and appendix patches of the payer. They include the sites for antigens to interact with lymphocytes.

- Secondary lymphoid organs serve as a sort of "monitoring station" for the contents of the extracellular fluids – such as the blood, lymph and tissue fluid.
- It is also the location where the lymphocytes are activated.
- Examples of secondary lymphoid organs include the spleen, tonsils, lymph nodes, and Peyer's patches.

1.Spleen

Spleen is a secondary lymphoid organ. It is solid, encapsulated organ located in the upper part of the abdominal cavity behind the stomach and close to the diaphragm. It is a component of lymphatic system. It contains efferent lymphatic vessels.It is mesodermal in origin.It is deep red in colour and has direct communication with the main arteriolar circulation. In contrast to lymph nodes which filter lymph, the spleen filters blood The spleen is surrounded by a capsule. The capsule penetrates into the tissues as septa called trubeculae.There are two distinct regions in the spleen, namely an



outer red pulp and an inner white pulp.

A. Red Pulp

- The red pulp consists of large numbers of blood-filled sinusoids in which phagocytes and plasma cells are found. The red pulp is concerned with the destruction of old and dead red blood cells and is a reserve site for spent erythrocytes.
- The red pulp is also reserve site for haematopoiesis. In the embryo, before the bone marrow starts producing the RBCs (till 5 months) the spleen is only concerned with the production of RBCs.
- In the adult, when there is a sudden demand for RBCs, for example during recovery from sudden anaemia, the spleen takes over the function of haematopoiesis temporarily, along with the bone marrow.

B. White pulp

- The white pulp consists of the lymphoid tissue. The cellular arrangement of the white pulp was first described by Malpighi hence it is also known as Malpighian follicle.
- The major part of the lymphoid tissue is arranged around a central arteriole and it is known as periarteriolar lymphatic sheath (PALS). The PALS consists of B lymphocytes grouped in small masses called follicles.
- In between the follicles are distributed the T lymphocytes The B lymphocyte follicle may also contain germinal centre, then it is known as secondary follicle.
- Surrounding the germinal centre is a layer of lymphocytes known as mantle layer. The PALS is separated from the red pulp by the marginal zone consisting of both T and B lymphocytes.
- The spleen traps the blood borne antigens and the initiation of both humoral and cellular immunity occurs in the spleen, in response to these antigens.
- Individuals who do not possess the spleen are readily susceptible to blood borne bacterial infections.

2. Lymph Node

- Lymph node, or lymph gland is a kidney-shaped organ of the lymphatic system, and the adaptive immune system.

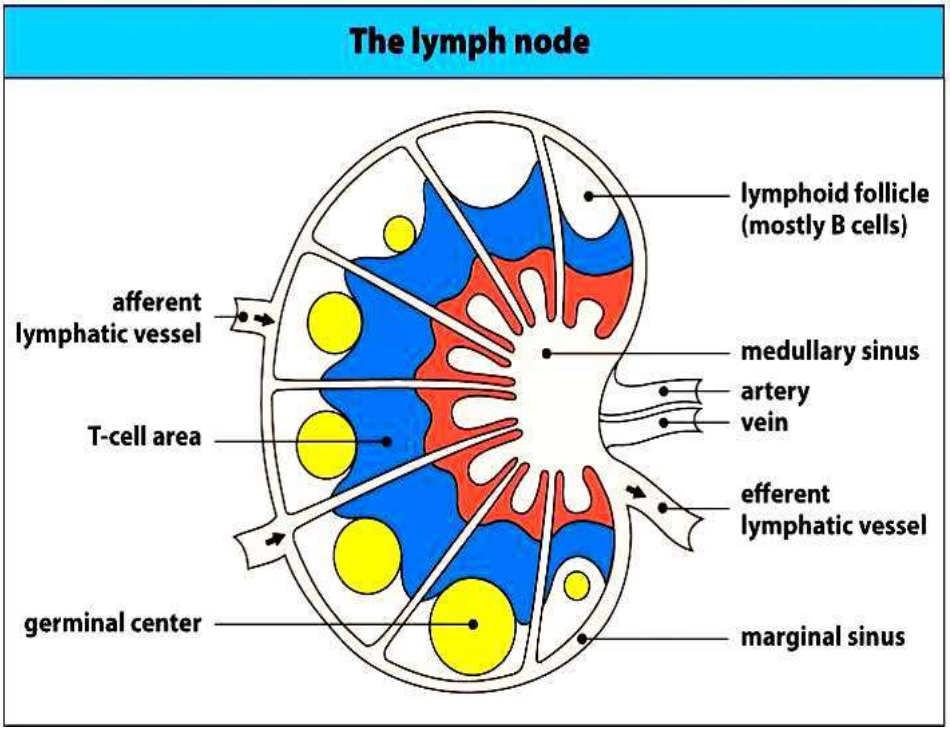
- A large number of lymph nodes are linked throughout the body by the lymphatic vessels. They are major sites of lymphocytes that include B and T cells.
- Lymph nodes are important for the proper functioning of the immune system, acting as filters for foreign particles including cancer cells, but have no detoxification function.
- Lymph nodes are kidney or oval shaped and range in size from 0.1 to 2.5 cm long. Each lymph node is surrounded by a fibrous capsule, which extends inside a lymph node to form trabeculae.
- The substance of a lymph node is divided into the outer *cortex* and the inner *medulla*. These are rich with cells.
- The hilum is an indent on the concave surface of the lymph node where lymphatic vessels leave and blood vessels enter and leave.
- Lymph nodes are present throughout the body, are more concentrated near and within the trunk, and are divided into groups.There are about 450 lymph nodes in the adult.

A. **Cortex and Medulla**

- A lymph node is divided into compartments called *nodules* (or lobules), each consisting of a region of cortex with combined follicle B cells, a para cortex of T cells, and a part of the nodule in the medulla. The substance of a lymph node is divided into the outer *cortex* and the inner *medulla*.
- The cortex of a lymph node is the outer portion of the node, underneath the capsule and the subcapsular sinus. It has an outer part and a deeper part known as the *paracortex*.
- The outer cortex consists of groups of mainly inactivated B cells called follicles. When activated, these may develop into what is called a germinal centre.
- The deeper paracortex mainly consists of the T cells. Here the T-cells mainly interact with dendritic cells, and the reticular network is dense. The medulla contains large blood vessels, sinuses and medullary cords that contain antibody-secreting plasma cells. There are fewer cells in the medulla.

B. Cells In the Lymph Node

- In the lymphatic system a lymph node is a secondary lymphoid organ. Lymph nodes contain lymphocytes, a type of white blood cell, and are primarily made up of B cells and T cells.
- B cells are mainly found in the outer cortex where they are clustered together as follicular B cells in lymphoid follicles, and T cells and dendritic cells are mainly found in the *paracortex*.
- There are fewer cells in the medulla than the cortex. The medulla contains plasma cells, as well as macrophages which are present within the medullary sinuses.
- As part of the reticular network, there are follicular dendritic cells in the B cell follicle and fibroblastic reticular cells in the T cell cortex. The reticular network provides structural support and a surface for adhesion of the dendritic cells, macrophages and lymphocytes.
- It also allows exchange of material with blood through the high endothelial venules and provides the growth and regulatory factors necessary for activation and maturation of immune cells.



UNIT-I (SHORT QUESTIONS)

1. PHAGOCYTOSIS

Phagocytosis is the process of engulfment and destruction of solid particles such as bacteria, dead tissue and foreign particles by the cells.

The cells performing phagocytosis are called phagocytes. The cell types are

- Neutrophils
- Monocytes and
- Macrophages

Process of phagocytosis can be explained in four steps-

Activation -

The first step also involves chemotaxis. Here, the cells move towards the area with a high concentration of the foreign particles/cells or molecules. Cells are chemically stimulated by the presence of the foreign molecules/ particles etc

Binding

Here, surface receptors on the phagocyte bind/adhere to the surface of the particle. This step of phagocytosis is necessary for the molecule to be ingested.

Depending on the cell, there are different types of surface receptors that play an important role in phagocytosis (binding).

- Scavenger receptors- Bind to various types of molecules on the surface of bacteria
- Opsonin receptors- Opsonin receptors are some of the most studied surface receptors. They bind to molecules that possess immunoglobulin G on their surface
- Antibodies- Some of the cells are capable of producing antibodies that make it possible to attach to certain antigens
- Toll-like receptors- These receptors bind to specific molecules on the surface of bacteria, fungi, and viruses

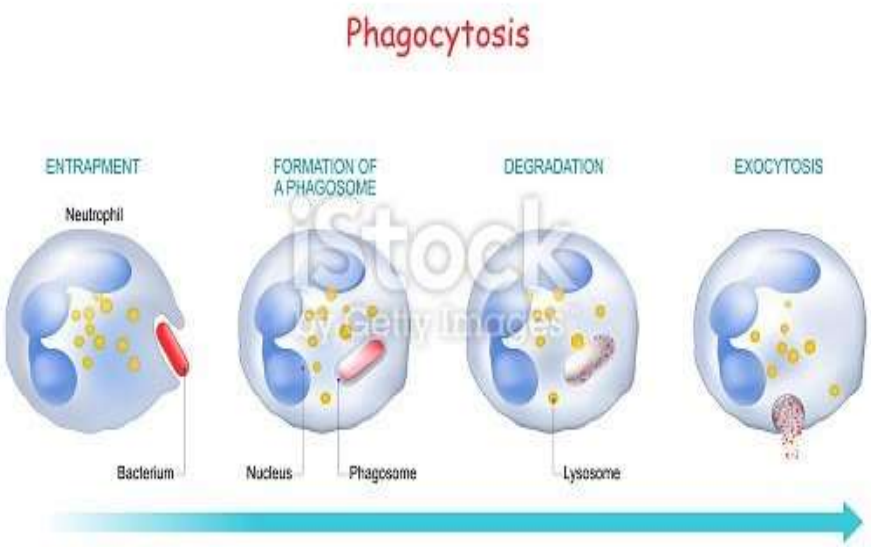
Receptor binding is an important step in phagocytosis allowing the cell to identify the object/particle/bacteria etc and thus elicit the appropriate response.

Ingestion

The cell (phagocyte) starts expanding as it surrounds the molecule. This process also involves formation of a vacuole or vesicle around the molecule as it is completely ingested.

Digestion

In some cells, enzymes in the vesicle (e.g. lysosome) break down the molecule into simpler components. Waste materials that cannot be used are then removed from the cell through a process known as exocytosis. However, for phagocytes involved in immunity, special structures are known as peroxisomes are created to trap and remove toxic molecules.



2. PASSIVE ACQUIRED IMMUNITY

Passive immunity is the resistance transmitted passively to an individual in a 'ready made' form.

- There is no antigenic stimulus; instead, preformed antibodies are administered.
- There is no latent period, protection being effective immediately.
- The immunity is transient, no secondary response in passive immunity.
- It is less effective then active immunisation.
- The protection is temporary lasting for few days
- The host does not produce any resistance.
- The host will not produce any antibodies or sensitised cells.
- The main advantage of passive immunisation is that it acts immediately and, therefore, can be used when immediate effect is desired, for example antidiphtheritic serum given to a child presenting with diphtheria.

Natural passive immunity

- It is the resistance passively transferred from mother to baby. In the human infants, maternal antibodies are transmitted predominantly through the placenta. It is only by the age of three months that the infant acquires some measure of immunological independence.
- The antibody crossing the placenta is Ig G, other immunoglobulins cannot pass the placenta.
- In some mammals antibodies enter the baby through colostrum i.e., the fist milk produced by the mother.
- The breast feed babies can absorb the antibodies directly from the gastrointestinal tract. Sometimes theses antibodies lie in the tract lining and send the microbes out.

Artificial passive immunity

It is the resistance passively transferred to a recipient by the administration of antibodies. This immunity is therapeutically used in the treatment of Tetanus, Diphtheria, snake bite and Immunodeficiency disease.

Artificial passive immunity can be brought about by using anyone of the following methods-

a. Hyperimmune serumof animal or Human origin-

The serum prepared from immunised man or animal by injecting an antigen is called Hyperimmune serum. The resistance developed in man or animal by repeated injection of antigen is called Hyperimmunization.

Anti-Tetanus serum is prepared by repeated administration of a tetanus toxoid to horses. The hyperimmune serum is then separated by bleeding the horses and the antibodies in the serum are separated, purified and given either intravenously or subcutaneously.

b. Convalescent Serum- serum collected from persons recovering from a particular infectious disease have high amounts of antibodies for the specific antigen causing that particular disease. We can collect their serum separate antibodies and use them to a weak immnunised person.

c. Polled sera of healthy individuals – in some communities we can see some diseases arising frequently. In those areas healthy person will have antibodies against those diseases. We can take antibosies from such persons and can inject into weak immunised persons.

3. TYPES OF VACCINES

Following are the different types of vaccines based on how they are made

- **Live attenuated vaccines**
  They have weakened form of the living viruses in them. They are the closest to natural infections hence they quite effective, but everyone cannot get them because people with weakened immune systems like those undergoing chemotherapy, may have adverse reactions. Examples include MMR, chickenpox.
- **Toxoid vaccines**
  These vaccines prevent diseases caused by bacteria that produce toxins (poisons) in the body. These have weakened forms of toxins called toxoids in them. For example, DTaP and Tdap vaccines contain diphtheria and tetanus toxoids, in addition to protection against pertussis.
- **Inactivated vaccines**

These have viruses in inactivated (killed) form. These vaccines produce immune responses, but cannot cause the disease itself. Examples include hepatitis A, influenza ,polio and rabies.

- **Subunit vaccines**
  They use only a part of the virus or bacteria instead of the full organism. They contain only the essential antigens from the causative agent; hence they cannot cause illness. An example is pertussis (whooping cough) component of the DTaP vaccine.
- **Conjugate vaccines**
  They use part of the coating of bacteria called polysaccharides. It is a type of subunit vaccine which combines a weak antigen with a strong antigen as a carrier so that the immune system has a stronger response to the weak antigen. Examples include hepatitis B, HPV, shingles, pneumococcal (PCV13), and meningococcal (MenACY)
- **Homologous vaccine**
  These vaccines are prepared against one microbe and are used against the infection of the same microbe.
- **Heterologous vaccine**
  These vaccines are prepared against one microbe and are used against the infection of different microbes.

4. ANTIGEN PRESENTING CELLS

- The innate immune system contains cells that detect potentially harmful antigens, and then inform the adaptive immune response about the presence of these antigens.
- An **antigen-presenting cell (APC)** is an immune cell that detects, engulfs, and informs the adaptive immune response about an infection.
- When a pathogen is detected, these APCs will phagocytose the pathogen and digest it to form many different fragments of the antigen.
- Antigen fragments will then be transported to the surface of the APC, where they will serve as an indicator to other immune cells.

Antigen-presenting cells fall into two categories:

1. Professional and 2. Non-professional.

1. Those that express MHC class II molecules along with co-stimulatory molecules and pattern recognition receptors are often called professional antigen-presenting cells.
2. The non-professional APCs express MHC class I molecules.

Professional APCs

Professional APCs specialize in presenting antigens to T cells. They are very efficient at internalizing antigens, either by phagocytosis (e.g. macrophages), or by receptor-mediated endocytosis (B cells), processing the antigen into peptide fragments and then displaying those peptides (bound to a class II MHC molecule) on their membrane. The T cell recognizes and interacts with the antigen-class II MHC molecule complex on the membrane of the antigen-presenting cell. An additional co-stimulatory signal is then produced by the antigen-presenting cell, leading to activation of the T cell. The expression of co-stimulatory molecules and MHC class II are defining features of professional APCs.All professional APCs also express MHC class I molecules as well.

The main types of professional antigen-presenting cells are dendritic cells, macrophages and B cells.

a.Dendritic cells (DCs)

Dendritic cells have the broadest range of antigen presentation and are necessary for activation of naive T cells.DCs present antigen to both helper and cytotoxic T cells. They can also perform cross-presentation, a process by which they present exogenous antigen on MHC class I molecules to cytotoxic T cells. Cross-presentation allows for the activation of these T cells.Dendritic cells also play a role in peripheral tolerance, which contributes to prevention of auto-immune disease.

Dendritic cells are present in those tissues that are in contact with the external environment, such as the skin (where there is a specialized dendritic cell type called the Langerhans cell) and the inner lining of the nose, lungs, stomach and intestines. They can also be found in an immature state in the blood. Once activated, they migrate to the lymph

nodes where they interact with T cells and B cells to initiate and shape the adaptive immune response. At certain development stages they grow branched projections, the dendrites that give the cell its name.

b.Macrophages

Macrophages are large, mononuclear phagocytic cells derived from monocytes. They are abbreviated as MΦ. They are distributed throughout the body,but they are concentrated in lymph nodes, spleen and liver.They are mononuclear and have large nucleus. They are agranulocytes and have long life span. They are strongly phagocytic.They can attach to the surface of cells hence called Adherent cells.

- At the affected site, the macrophage surrounds the site of infection or tissue damage with its membrane in a mechanism called phagocytosis.
- Macrophages can be stimulated by T cell secretion of interferon. After this activation, macrophages are able to express MHC class II and co-stimulatory molecules, including the B7 complex and can present phagocytosed peptide fragments to helper T cells.

c. B cells

B cells, also known as B lymphocytes, are a type of white blood cell of the lymphocyte subtype. They function in the humoral immunity component of the adaptive immune system. B cells produce antibody molecules which may be either secreted or inserted into the plasma membrane where they serve as a part of B-cell receptors.

When a naïve or memory B cell is activated by an antigen, it proliferates and differentiates into an antibody-secreting effector cell, known as a plasmablast or plasma cell. Additionally, B cells present antigens (they are also classified as professional antigen-presenting cells (APCs)) and secrete cytokines.

In mammals, B cells mature in the bone marrow, which is at the core of most bones. In birds, B cells mature in the bursa of Fabricius, a lymphoid organ where they were first discovered by Chang and Glick,which is why the 'B' stands for bursa and not bone marrow as commonly believed.

B cells, unlike the other two classes of lymphocytes, T cells and natural killer cells, express B cell receptors (BCRs) on their cell membrane.]BCRs allow the B cell to bind to a foreign antigen, against which it will initiate an antibody response.

Non-professional Antigen presenting cells

Non-professional antigen presenting cells include all nucleated cell types in the body. They use an MHC class I molecule coupled to beta-2 microglobulin to display endogenous peptides on the cell membrane.

5.THYMUS

Thymus is a primary lymphoid organ. Thymus is the training centre for the army of T lymphocytes. It resembles a thyme leaf and hence the name. It is a derivative of pharynx.It develops from the epithelium of the 3rd and 4th pharyneal region.It begins its development on the 6th week of gestation.

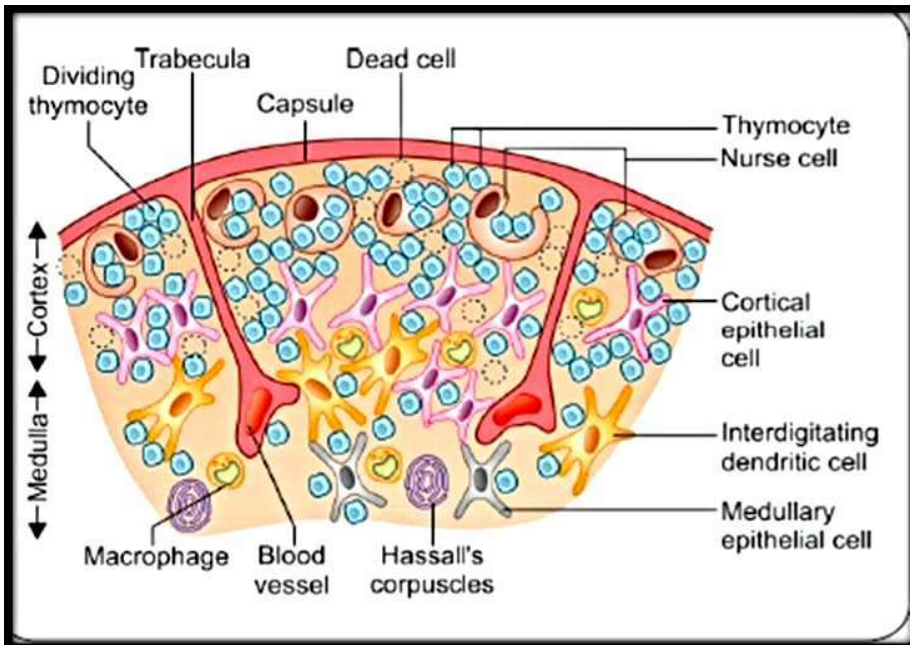
- At the time of birth, it weighs about 15 to 20 gms and it m after birth. It reaches about 40 gms in weight by puberty and there atrophies..In human beings, the thymus consists of two oval lobes just be of the sternum, below the thyroid gland. In birds it is made up of 7 lobes on each side of the neck.
- The thymus is covered by a fibrous capsule. It is formed of two lobes. Each lobe of the thymus is organized into lobules which are separated from one another by septa called trabeculae.
- Within each lobule, the cells are arranged into an outer cortex and innermedulla.The cortex is tightly packed with proliferating immature lymphocytes. The cortex consists of lymphocytes (thymocytes) and reticular cells.
- The reticular cells are larger than lymphocytes and they form a dimensional network and in the meshes of which the lymphocytes are found.
- The medulla consists of vascular tissue, reticular epithelial cells and scattered lymphocytes. There are also some interdigitating cells associated with the epithelial network and these cells are rich in MHC class II antigens.

- In addition, there are some peculiar structures in the medulla called Hassall's corpuscles. These are small masses or whorls of epithelial cells around a central degenerating epithelial cell, their function is not known.
- The T lymphocytes are non-functional when they are inside the thymus, as they do not come in contact with the antigen
- The non-execution of the immune response is due to the epithelial barrier which prevents the antigens of the blood to contact the lymphocytes of the thymus.
- These non-functional T lymphocytes travel through the blood and lymph circulation and ultimately reach the thymus dependent areas of the peripheral lymphoid organs and get colonized there. In these organs, these T lymphocytes become functional by antigenic stimulation.

Functions of Thymus

1. T lymphocytes mature in the thymus.
2. It brings about cell mediated immunity.
3. It brings about graft rejection.

T.S of Thymus



6. LESS ORGANISED SECONDARY LYMPHOID ORGANS

Secondary lymphoid organs are two types-

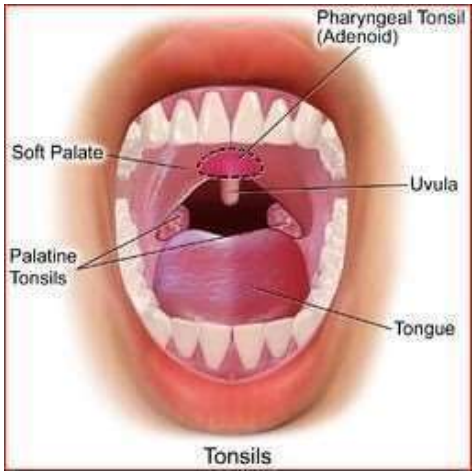
a. Well organised secondary lymphoid organs-Spleen and Lymph nodes
b. Less organised secondary lymphoid organs –MALT,GALT,BALT.Tonsils , Payers patches

1. **Mucosa-associated lymphoid tissue (MALT)-** consists of an aggregate of lymphoid follicles directly associated with the mucous membrane epithelia. MALT makes up dome-shaped structures found underlying the mucosa of the gastrointestinal tract, breast tissue, lungs, and eyes. Peyer's patches, a type of MALT in the small intestine, are especially important for immune responses against ingested substances. Peyer's patches contain specialized endothelial cells called M (or microfold) cells that sample material from the intestinal lumen and transport it to nearby follicles so that adaptive immune responses to potential pathogens can be mounted.

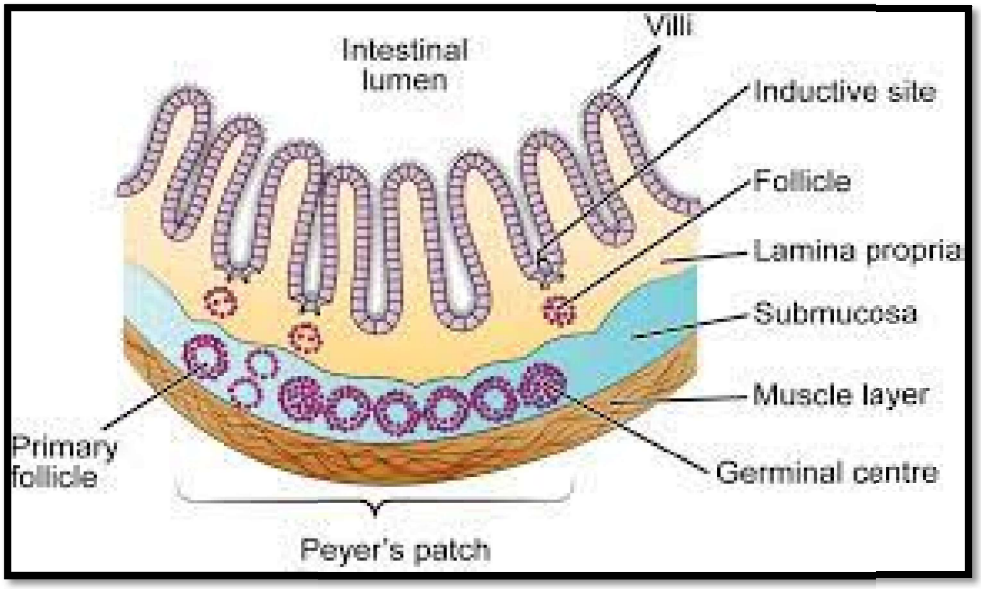
2. **BALT- Bronchus-associated lymphoid tissue -**consists of lymphoid follicular structures with an overlying epithelial layer found along the bifurcations of the bronchi, and between bronchi and arteries. They also have the typically less-organized structure of other lymphoid nodules. These tissues, in addition to the tonsils, are effective against inhaled pathogens

3. **GALT- Gut-associated lymphoid tissue** (GALT)**-** is a component of the mucosa-associated lymphoid tissue (MALT) which works in the immune system to protect the body from invasion in the gut.

4. **Tonsils** - Tonsils are lymphoid nodules located along the inner surface of the pharynx and are important in developing immunity to oral pathogens. The tonsil located at the back of the throat, the pharyngeal tonsil, under the tongue are Lingual tonsils and above the roof palatins tonsils. Thesestructures, which accumulate all sorts of materials taken into the body through eating andbreathing, actually "encourage" pathogens to penetrate deep into the tonsillar tissues where theyare acted upon by numerous lymphoid follicles and eliminated.


Pharyngeal Tonsil
(Adenoid)
Soft Palate
Uvula
Palatine
Tonsils
Tongue
Tonsils

GALT


Intestinal
lumen
Villi
Inductive site
Follicle
Lamina propria
Submucosa
Muscle layer
Primary
follicle
Germinal centre
Peyer's patch

IMMUNOLOGY UNIT-II NOTES

(ESSAY QUESTIONS)

I . ANTIGEN AND ITS PROPERTIES

Definition

Antigen is a substances usually protein in nature and sometimes polysaccharide, that generates a specific immune response and induces the formation of a specific antibody or specially sensitized T cells or both. Although all antigens are recognized by specific lymphocytes or by antibodies, only some antigens are capable of activating lymphocytes. Molecules that stimulate immune responses are called Immunogens.

Autoantigens- for example, are a person's own self antigens. Examples: Thyroglobulin, DNA, Corneal tissue, etc.
Alloantigens - are antigens found in different members of the same species (the red blood cell antigens A and B are examples).
Heterophile antigens- are identical antigens found in the cells of different species. Examples: Forrssman antigen, Cross-reacting microbial antigens, etc.

Properties of antigen

1. Autoantigens

- An autoantigen is usually a normal protein or complex of proteins (and sometimes DNA or RNA) that is recognized by the immune system of patients suffering from a specific autoimmune disease
- These antigens should not be, under normal conditions, the target of the immune system, but, due mainly to genetic and environmental factors, the normal immunological tolerance for such an antigen has been lost in these patients.
- Examples: Nucleoproteins, Nucleic acids, etc.

2.Antigen Specificity

- Antigen Specificity depends on the specific actives sites on the antigenic molecules (Antigenic determinants).
- Antigenic determinants or epitopes are the regions of antigen which specifically binds with the antibody molecule.

3. Species Specificity

- Tissues of all individuals in a particular species possess, species specific antigen.
- Human Blood proteins can be differentiated from animal protein by specific antigen-antibody reaction.

4. Organ Specificity

- Organ specific antigens are confined to particular organ or tissue.
- Certain proteins of brain, kidney, thyroglobulin and lens protein of one species share specificity with that of another species.

5. Auto-specificity

The autologous or self antigens are ordinarily not immunogenic, but under certain circumstances lens protein, thyroglobulin and others may act as autoantigens.

6. Isospecificity

Isospecificity is determined by the presence of isoantigens or histocompatibility antigens.Isoantigens are antigens found in some, but not all, members of a species. A species may be grouped depending on the presence of different isoantigens in its members. These are geneti-cally determined. Human erythrocyte antigens, based on which individuals are classified into different blood groups, are the best examples of isoantigens in humans. The blood groups are of primary importance in:

- Transfusion of blood and blood products,
- Isoimmunization during pregnancy

II.STRUCTURE OF IMMUNOGLOBULIN / ANTIBODIES

Introduction

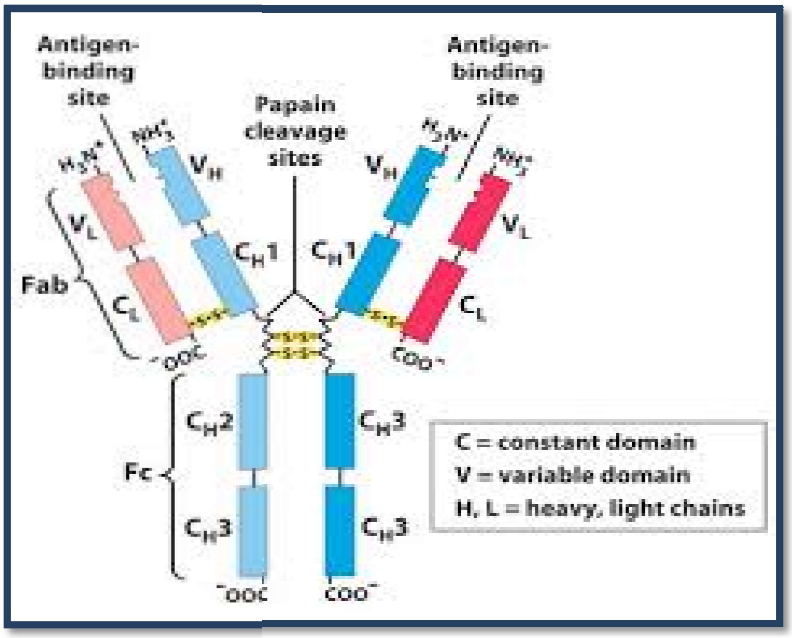
Antibodies, also known as immunoglobulins, are proteins produced by lymphocytes as a result of interaction with antigens. Antibodies are a part of the humoral immune of the adaptive immune system where each antibody identifies a specific antigen and protects the body against it.

- Antibodies are glycoproteins that bind to antigens with a high degree of specificity and affinity.
- B lymphocytes are stimulated by the binding of antigen, which results in the secretion of millions of antibodies in the bloodstream.
- The produced antibodies circulate through the bloodstream and neutralize antigens that are identical to those that triggered the immune response.
- The binding of antibodies to microorganisms or other such antigens can result in the microorganism being immobile or preventing them from penetrating the cells.
- Antibodies carry out two principal functions in the immune system. The first function is the recognition and binding to foreign bodies. The second more important function is to trigger the elimination of the attached foreign material.
- Since millions of antibodies are produced during an immune response, some of these remain in circulation in the blood for several months. This provides an extended immunity against the particular antigen.
- Each antibody is a Y-shaped protein where each tip of the Y contains a paratope that recognizes an epitope of a particular antigen.
- Antibodies can be classified into different classes based on different structures and functions.

Basic Structure Of Immunoglobulin /antibodies

- Antibodies are globular plasma proteins with a basic Y-shaped structure and four polypeptides.
- There are two identical heavy chains and two identical light chains connected together by disulfide bonds. The light chains consist of polypeptides of the size 22,000 Da, and the heavy chains consist of polypeptides of the size 50,000 Da.

- Each heavy chain is connected to a light chain by a disulfide bond, and the two heavy chains are connected to the light chains by two sulfide bonds.
- There are five different types of heavy chains in mammals that are designated by letters: α, δ, γ, ε and μ. There are two types of light chains designated by λ and κ.
- An antibody is composed of a variable region and a constant region. The variable region changes to various structures depending on the differences in the antigens. The constant regions are constant and do not change with antigens.
- The variable region varies between clones and is involved in antigen recognition. The constant region is conserved among clones and is required for the structural integrity and effector functions.
- The heavy and light chain in an immunoglobulin molecule consists of an amino-terminal variable region with 100-110 amino acids.
- Each heavy chain has one variable domain and one constant domain. The light chain, in turn, consists of one variable domain and three constant domains.
- The rest of the chain consists of the constant region, which limited variation that determines the light chain subtypes.
- The heavy chains in some antibodies contain a proline-rich hinge region. The hinge region separates the antigen-binding and effector domains.
- The region allows the movement of the domains enabling them to bind to antigens separated by varying distances.



III. EPITOPES

- An epitope, also known as antigenic determinant, is the part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or T cells. For example, the epitope is the specific piece of the antigen to which an antibody binds.
- The part of an antibody that binds to the epitope is called a paratope. Although epitopes are usually non-self proteins, sequences derived from the host that can be recognized (as in the case of autoimmune diseases) are also epitopes.
- An antibody that is specific for an antigen binds non-covalently to a region of the molecule surface known as epitope. Naturally occurring epitopes are relatively small (either amino-acids or sugar residues).Specific epitope should fit with the specific site present on antibody (antibody-binding site).
- The site present on antibody called antigen- combining site or paratope is a cave pocket shaped one to match with the epitope having a convex site. Small antigens are mainly mono-epitopic where as large proteins and oligosaccharides can express many different and/or identical repeating multi-epitopes.
- The forces responsible for binding include hydrophobic and Vander Waals forces, which are spherical, symmetrical and hydrogen bridges, which are directional and require matching of the reactants. Electrostatic forces might also contribute, but they act at distance.
- Formation of stable immune complexes normally occur only when the epitope and paratope. Not only the position of on epitope within a large molecule is important in determining its ability toinduce an immune response, but the position of each subunit within the epitope may also be important.
- For e.g. each of the amino acid residues comprising a given accessible epitope might unequally contribute to bind with an antibody paratope. Thus some components of an epitope are more immuno-dominant than others.

B cell Epitopes-

- See on the exposed surface of the Immunoglobulin.
- They are soluble antigen and tend to be accessible.
- B cell epitopes generally contain hydrophilic aminoacids

- Often the epitopes are found where the molecule bends are where there is high degree of segmental mobility.
- Complex proteins contain multiple overlapping epitopes. The size of the Bcell epitope is determined by the size of the antigen site on the antibody molecule.
- Larger globular antigen tend to interact with Ig across a larger face.
- His type of interation is obviously highly dependant upon the 3D confirmation of the globular antigen.

T cell Epitopes –

- T cell epitopes are presented on the surface of an antigen presenting cell, where they bound the MHC molecules.
- T cell epitopes presented by the MHC class I molecules are typically peptide between 8 and 11 amino acid in length whereas Class II molecules are present non-peptidic epitope such as glycolipic.
- T cell antigen often contain amphipathic peptide containing both a hydrophilic and hydrophobic region. The hydrophobic region serves as a agrotope while the hydrophilic region serves as the epitope.
- Immunodeterminent T cell epitope are determined by the set of MHC molecules which are exposed by an individual.

IV. ENDOGENOUS PATHWAY

Introduction

- The degradation of antigens into peptide fragments, combining them with MHC molecules into peptide - MHC complexes and depositing them on the surface of cells constitute antigen processing and presentation.
- The degradation of antigen is called antigen processing .The deposition of degraded antigen on the surface of the cell for the identification of T cells is called antigen presentation.
- The APCs are the spies of the immune system. They keep a watch on the pathogens (enemies).When they come across a pathogen, they swallow it, degrade it into peptide fragments and display a bit of the fragment on their surface. Then the APC tells the T cell "I have identified an enemy; I have captured him and I have his identity on my hand; Go and kill him"

MHC –I molecule structure

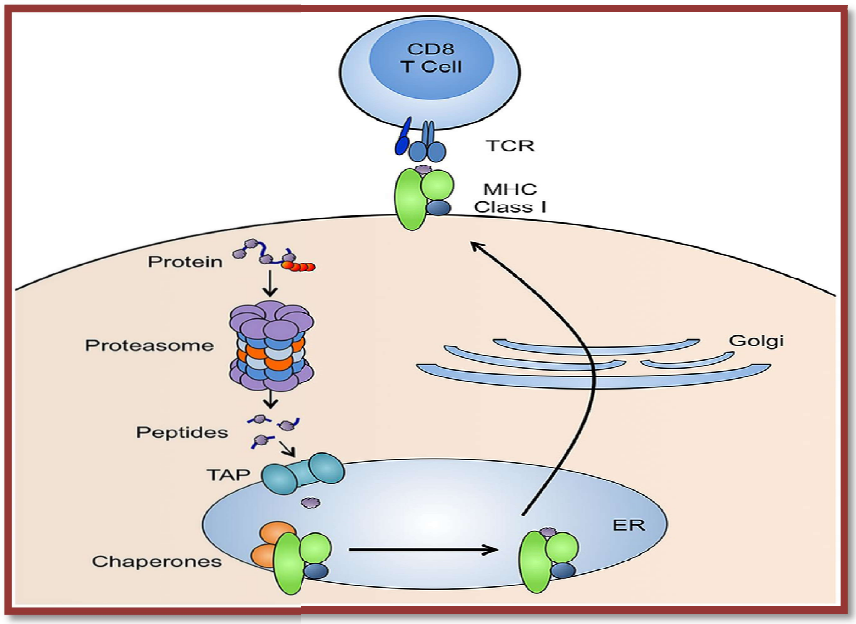
- The structure of Class I MHC molecule consists of two polypeptide chains α and β. These two chains are connected together by non-covalent bonds. The α chain is characterized as an internal membrane glycoprotein with a molecular weight of 45000 Da (in humans). B chain, on the other hand, is an extracellular microglobulin with a molecular mass of 12kDa.
- The α chain is made up of approximately 350 amino acids and also divided into three globular domains α_1, α_2 and α_3. Each of these domains contains roughly 90 amino acids. The α_1 and α_2 domains interact to form peptide-binding units of class I MHC molecule.
- Moreover, α chain also consists of a stretch of 26 hydrophobic amino acids which holds the α chain on the plasma membrane.
- T_{cyt} Cell (cytotoxic T cell) has specificity towards cells containing peptides associated with Class I MHC due to the presence of CD8 antigen on the surface of Tcyt Cell. CD8 antigen has an affinity towards the α_3 domain of Class I MHC molecules.

Endogenous pathway/ Cytosolic pathway

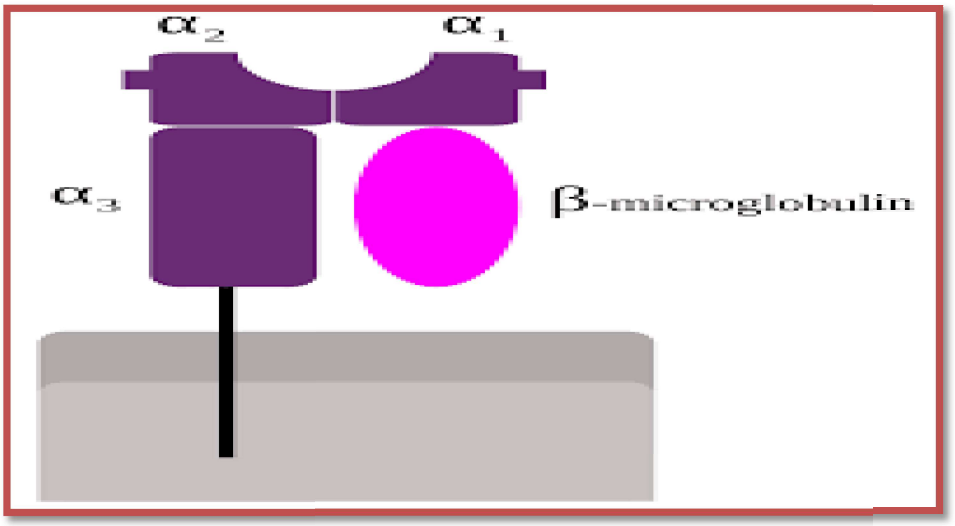
- Class I MHC molecules involve in presenting intracellular or endogenous pathogens or antigens. Intracellular pathogens refer to those organisms which live and replicates inside the host cell. An example of this type of pathogen is a virus.
- Under normal condition the MHC class I molecules forms a complex with the self-peptides or self-antigens. While, in case of any viral infection, the MHC class I molecules present the peptide derived from the virus which is then further recognized by T cells.
- Cell components such as a nucleus, endoplasmic reticulum and Golgi apparatus play an important role in antigen processing and presentation.
- When a virus infected a normal cell, the viral DNA moves inside the cell and produce viral proteins with the help of the host cell mechanisms. The viral proteins are synthesized in the cytosol.
- The cytoplasm also contains a cylindrical protein complex called the proteasome. The main function of the proteosome is to degrade the unwanted or damaged protein into smaller peptides.

- At the time of viral infection, the viral proteins interacted with the proteosomes present in the cytoplasm. The processing took place in the cytosol and as a result, the proteins are degraded into smaller peptides (8-15 amino acid long).
- In the next step, these fragmented peptides are transported into the endoplasmic reticulum. The transport took place due to a peptide delivery system called the transporter associated with antigen processing (TAP). TAP is made up of two domains or subunits called TAP 1 and TAP 2.
- Inside the endoplasmic reticulum the α and β chains of MHC class I molecules are synthesized and by the help of a group of chaperone proteins, the MHC class I molecule is formed and moves towards the TAP. As a result, the peptides bind at the peptide-binding site of the class I MHC molecule inside the endoplasmic reticulum and forms the MHC class I-peptide complex.
- In the next step, the MHC class I- peptide complex moves to the surface of the Golgi apparatus and by the help of secretory vesicle, it moves towards the surface of the plasma membrane.
- Once the MHC class I-peptide complex reaches the cell surface, the T cell receptors recognize the antigen peptide complex. Moreover, the co-receptor CD8 of the T cell attaches with the α_3 domain of the MHC class I molecule. Hence, the antigen is presented to the T cell.

Endogenous Pathway



MHC-I molecule Structure



V.STRUCTURE OF MHC-CLASS II MOLECULE AND EXOGENOUS PATHWAY

Introduction

- The degradation of antigens into peptide fragments, combining them with MHC molecules into peptide - MHC complexes and depositing them on the surface of cells constitute antigen processing and presentation.
- The degradation of antigen is called antigen processing .The deposition of degraded antigen on the surface of the cell for the identification of T cells is called antigen presentation.
- The APCs are the spies of the immune system. They keep a watch on the pathogens (enemies).When they come across a pathogen, they swallow it, degrade it into peptide fragments and display a bit of the fragment on their surface. Then the APC tells the T cell "I have identified an enemy; I have captured him and I have his identity on my hand; Go and kill him"

MHC-II molecule structure

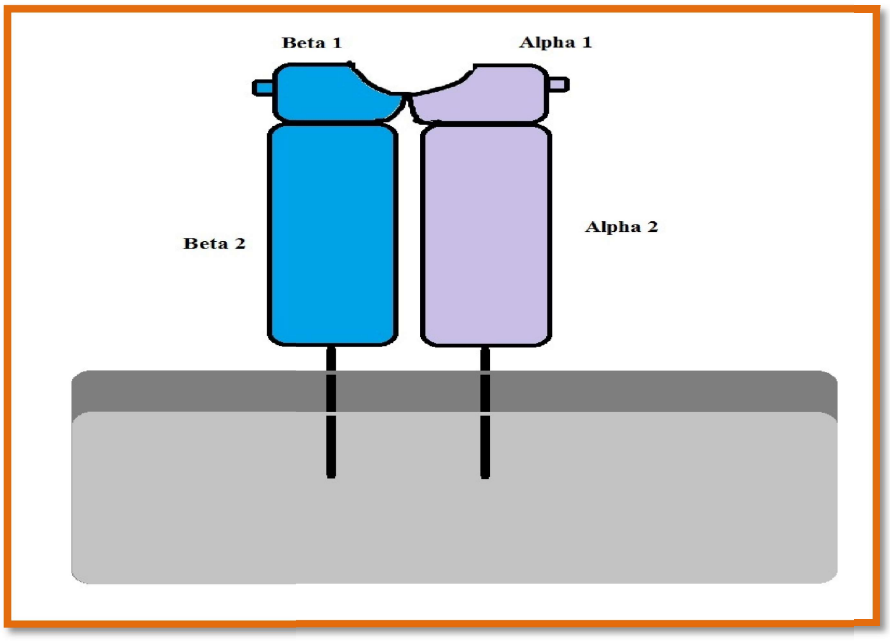
- Class II MHC molecules are heterodimers and characterized by two non-covalently connected polypeptide chains. The chains are termed a heavy chain (α, 30kDa) and light chain (β, 26kDa).
- Similar to class I MHC molecules, class II MHC molecules are also characterized by an extracellular amino-terminal domain, a transmembrane domain, and an intracellular carboxy-terminal tail.
- The class II MHC molecules are expressed on the surface of the antigen-presenting cells such as B cells, dendritic cells, and macrophages.
- The α chain is divided into two domains α_1 and α_2, while the β chain is also divided into two groups β_1 and β_2. The β_2 domain is responsible for the binding of T cell co-receptor CD4.
- The α_1 and β_1 domains, on the other hand, are involved in the formation of the antigen-binding sites. Peptides containing 13-20 amino acids can bind at the antigen-binding site of class II MHC.
- The presence of disulfide bonds in α_2, β_1, and β_2 domains is also an important structural feature of the class II MHC molecules.

Exogenous Pathway

- MHC class II molecules are responsible for presenting exogenous or extracellular pathogen or antigen. The extracellular pathogen refers to the organisms which can grow and reproduce outside of the host cell. Bacteria, exotoxins, parasites are examples of extracellular antigens. These antigens are taken up by the cell by endocytosis or phagocytosis.
- Only the antigen-presenting cells involved in antigen processing and presentation by MHC class II molecules. These cells include B cells, macrophages, and dendritic cells. The pathway took place only after the engulfment of the antigen by the antigen-presenting cells.
- Inside the cell, the antigen carries a covering called an endosome. The endosome is fused with the lysosome present in the cytoplasm and forms endolysosomes. As a result, the foreign protein is degraded by the proteolytic enzyme present inside the lysosome and small peptides are formed.

- The class II MHC molecules are synthesized and formed in the endoplasmic reticulum. The α and β chain of the molecule is also associated with the invariant chain. This association helps to restrict the binding of self-antigen with the class II MHC molecule. The invariant chain- MHC complex is then transported from the endoplasmic reticulum to the Golgi apparatus and from the Golgi apparatus to another vesicle. Inside the vesicle, the invariant chain is digested and only a small fragment (Class II-associated invariant chain polypeptide: CLIP) is attached with the molecule.
- In the next step, the vesicle containing the MHC class II molecule is then fused with the vesicle containing fragmented peptides. The fragmented peptide is then bound with the MHC class II molecule by displacing the CLIP. This newly formed MHC class II-peptide complex is then transported to the surface of the cell.
- Once at the cell surface, the antigen is presented to the T cells. The T cell recognizes the peptide bound with the MHC class II molecule by the help of the T cell receptor and the CD4 co-receptor binds with the β_2 domain of the class II MHC molecule.

MHC-II molecule



VI. TWO HYPERSENSITIVE REACTIONS

Hypersensitivity (also called hypersensitivity reaction or intolerance) refers to undesirable reactions produced by the normal immune system, including allergies and autoimmunity. They are usually referred to as an over-reaction of the immune system and these reactions may be damaging, uncomfortable, or occasionally fatal. Hypersensitivity reactions require a pre-sensitized (immune) state of the host. Several types of hypersensitive reactions can be identified, reflecting differences in the effector molecules generated in the course of the reaction.

They are classified in four groups after the proposal of P. G. H. Gell and Robin Coombsin 1963.Gell and Coomb described four types of hypersensitivity reactions (Types I, II, III and IV). The first three types are antibody-mediated and the fourth type is mediated mainly by T-cell and macro-phases i.e. cell-mediated.

1. Type I Hypersensitivity:

Type I hypersensitive reactions are the commonest type among all types which is mainly induced by certain type of antigens i.e. allergens. Actually anaphylaxis means “opposite of protection” and is mediated by IgE antibodies through interaction with an allergen.

Anaphylaxis is defined as an allergic reaction of an organism to a foreignsubtance to which it has previously become sensitized resulting from the releasea of histamine, serotonin and other vasoactive substances. The term anaphylaxis was coined by Richet in 1902 and it means 'without protection.

Anaphylaxis is an immediate type hypersensitivity. Anaphylaxis is a severe life-threatening allergy. It must be treated as amedical emergency.In anaphylaxis, antibodies are fixed on the surface of mast cell and basophils sensitized host. The antigen combines with the cell fixed antibody, leading to release of pharmacologically active substances (vasoactive amines) which produce the clinical reaction. They affect skin, heart and respiratory system.

Factors causing Anaphylaxis-

Almost any substance can induce anaphylaxis, but the most common agents are drugs such as penicillin, foods such as nuts and shellfish, and insect venom. Anaphylaxis may occur after contact with extremely small amounts of antigen and is more common in persons with a history of atopic dermatitis.

Mechanism of Anaphylaxis –

The culprit of anaphylactic hypersensitivity is one type of immunoglobulin called IgE antibody.When a person receives the allergens (antigens) for the first time, the allergens get attached to the B cells. The allergens stimulate the B cells to proliferate into plasma cells. The plasma cells make IgE antibodies.

The IgE antibodies are called reagins and they are made by people who are allergic. The reaginic antibodies have a strong affinity for fixation to mast cells or basophils in skin or mucous surfaces.

The IgE antibodies produced for the first time, get attached to the surface receptors of mast cells with the help of their Fc fragment. This reaction would not harm the person and the person is now said to be immunized or sensitized As this initial contact with antigen leads to the priming of the B cells, this is called primary dose.

When the animal is exposed to the same antigen for the second time then he would be in danger. The IgE antibodies attached to the surface of mast cells bind with the antigens. The allergens cross-link the IgE antibodies attached to the mast cells. Subsequent contact with the allergen causes manifestations of hypersensitivity. This is known as shock - dose.

This cross -linking of IgE antibodies triggers the mast cells and a series of enzymatic reactions occurs inside the mast cells. As a result the mast cells release granules. The phenomenon of releasing of granules from mast cells is called degranulation. These granules contain substances like histamine,

serotonin, heparia, etc. These substances are the primary cause for anaphylaxis.

Therapy for Type-I hypersensitivity:

1. The first step in controlling type I is to identify the offending allergen and avoid contact if possible.

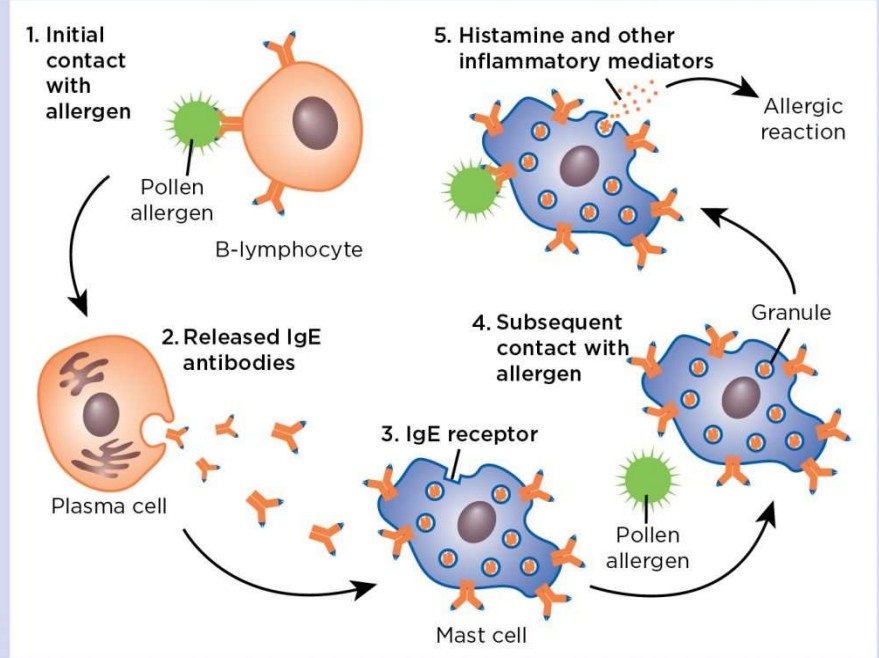
2. Removal of house pets, dust-control measures.

3. Repeated injections of increasing doses of allergens called hypo sensitization.

4. Enhancement of phagocytosis by IgG antibody which is referred to a blocking antibody because itcompetes for the allergens, binds and forms a complex that can be removed by phagocytosis.

5. Successful use of anti-histamine drugs result better with respect to type I hypersensitivity.

Fig 1. **IgE-mediated allergic response**



Type II Hypersenstivity

Type II hypersensitive reactions are those in which tissue or cell damage is the direct result of the actions of antibody and complement.

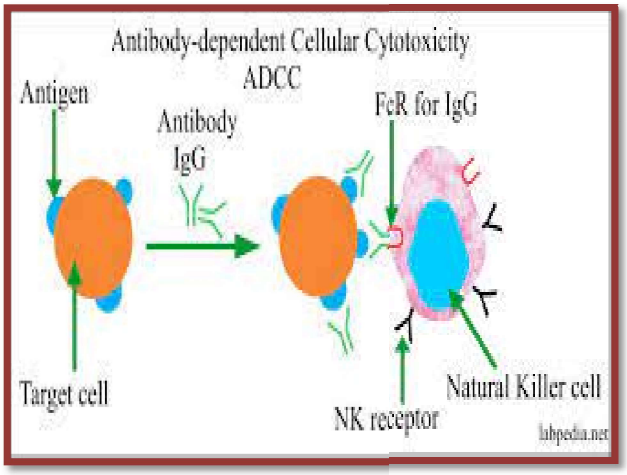
(i) Mode of action:

This type of reaction is resulted by blood- transfusion reactions in which host antibodies react with foreign antigens present on the incompatible transfused blood cells and mediate destruction of these cells.Antibody can mediate cell destruction by activating the complement system to create pores in the membrane of the foreign cell by forming membrane attack complex (MAC). This can also be mediated by antibody dependent cell-mediated cytotoxicity (ADCC).

A faulty cross-matching leads to haemolysis of the donor's erythrocytes in the blood vessels of the recipient due to the alloantigen of the donor's erythrocytes react with the antibodies in the serum of the recipient and in combination with activated complement, the erythrocytes undergo haemolysis.

(ii) Biological effect:

1. Haemolytic disease of the newborn develops when maternal IgG antibodies specific for foetal blood-group antigens cross the placenta and destroy foetal red blood cells. Severe haemolytic disease of the new born is called erythroblastosis foetalis, when an Rh+ foetus expresses an Rh antigen on its blood cells that the Rh– mother does not express it.



2. Certain antibiotics (e.g. penicillin, cephalosporin and streptomycin) can absorb non- specifically toproteins on RBC membranes, forming a complex similar to a hapten-carrier complex and gradually induces anaemia called drug-induced haemolytic anaemia

UNIT-II (SHORT QUESTIONS)

1. EPITOPES

An epitope, also known as antigenic determinant, is the part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or T cells. For example, the epitope is the specific piece of the antigen to which an antibody binds. The part of an antibody that binds to the epitope is called a paratope. Although epitopes are usually non-self proteins, sequences derived from the host that can be recognized (as in the case of autoimmune diseases) are also epitopes. An antibody that is specific for an antigen binds non-covalently to a region of the molecule surface known as epitope. Naturally occurring epitopes are relatively small (either amino-acids or sugar residues).Specific epitope should fit with the specific site present on antibody (antibody-binding site).

B cell Epitope-

A B-cell epitope is the antigen portion binding to the immunoglobulin or antibody. These epitopes recognized by B-cells may constitute any exposed solvent region in the antigen and can be of different chemical nature. However, most antigens are proteins and those are the subjects for epitope prediction methods.

- See on the exposed surface of the Immunoglobulin.
- They are soluble antigen and tend to be accessible.
- B cell epitopes generally contain hydrophilic aminoacids
- Often the epitopes are found where the molecule bends are where there is high degree of segmental mobility.
- Comp lex proteins contain multiple overlapping epitopes. The size of the Bcell epitope is determined by the size of the antigen site on the antibody molecule.
- Larger globular antigen tend to interact with Ig across a larger face.
- His type of interation is obviously highly dependant upon the 3D confirmation of the globular antigen.

T Cell Epitope

- T cell epitopes are presented on the surface of an antigen presenting cell, where they bound the MHC molecules.
- T cell epitopes presented by the MHC class I molecules are typically peptide between 8 and 11 amino acid in length whereas Class II molecules are present non-peptidic epitope such as glycolipic.
- T cell antigen often contain amphipathic peptide containing both a hydrophilic and hydrophobic region. The hydrophobic region serves as a agrotope while the hydrophilic region serves as the epitope.

2. FACTORS INFLUENCING IMMUNOGENICITY

Immunogens play a pivotal role in determining the status of immune system. Immune system always try to recognize foreign invaders and also try to get rid of antigenic effect.
Somehow, the body must recognize a foreign substance in order to evoke an immune response:

There are some essential factors which influence the power of antigen

Those are:

i. Molecular size,

ii. Structural stability,

iii. Degradability,

iv. Foreignness,

v. Chemical composition and heterogeneity,

vi. Antigen processing and presentation,

vii. Conformation and

viii. Accessibility.

i. Molecular size:

In general, large molecules are better antigens than small molecules. There is a direct correlationbetween the size of molecules and immunogenicity.As for example, hemocyanin is an invertebrate blood protein with (6.7 x 103)kDa molecular size, is apotent antigen in nature. Serum albumin from other mammals (69 kDa) is a fairly good antigen butmay also provoke tolerance, whereas the hormone angiotensin (1031 Da) is a poor antigen.Sometimes, very small molecules may bind to large proteins and resulting in formation of activeantigen which can evoke an immune system. The best immunogens tend to have a molecular massapproaching 100000Da.

ii. Structural stability:

The recognition of a molecule or part of a molecule as foreign is possible by the cells of the immunesystem due to its specific shape. Those molecules are recognized as poor antigens which lack aspecificor fixed shape. As for example gelatin is recognized as a poor protein due to its structural instability.This is being stabilized by cross-linking of the peptide chain with tyrosine or tryptophan.The major protein of bacterial flagellum called flagellin is a weak antigen due to its unstablestructure;it is being enhanced by polymerization. Proteins are much more stable antigens than starch(polysaccharide), lipids, carbohydrates and nucleic-acids.

iii. Degradability:

Easy degradation of an antigenic molecule is considered as an important factor with respect to theirantigenicity. The cells of the immune system recognize small molecular fragments and soluble antigens. When a molecule does not undergo breakdown process, itcan not be considered as antigen. For e.g., stainless steel pins and plastic joints are commonly implanted in the body without triggering an immune response. Different metals or organic polymers, plastic cannot be fragmented and processed to form suitable for triggering an immune response. Conversely, since immune responses are antigen driven, foreign molecules are very rapidly destroyed immune response.

iv. Foreignness:

The first and foremost criteria for a molecule to function as an immunogen is that it must be or act as non-self to the host. The cells, whose function is to respond to antigen (antigen-sensitive cells) areselected in such a way that they do not usually respond to normal body components. The degree of immunogenicity depends upon the degree of foreignness of the immunogen. When an antigen is introduced into an organism, greater the phylogenetic distance between two species, thegreater the genetic (i.e. antigenic) disparity between them. But all foreign substances donotelicitimmune response.As for e.g. carbon granules evoke phagocytosis but notantibody production. But the bovine serumalbumin (BSA) is an excellent immunogen when it is injected into a rabbit or other mammals but notat all an immunogen when it is introduced within the blood of cow itself. A kidney graft from an identicaltwin will be readily accepted but a graft from an unrelated human will be rejected in about twoweeks and a graft from a chimpanzee to a human will be rejected within a few hours due to disparityof protein structure and firmness with respect to evolutionary stand point view.

v. Chemical composition and heterogenity:

Not only molecular size, structural stability and foreignness but also chemical composition of an immunogen is an effective factor which affects its immunogenicity. As for e.g. artificial or synthetic chomo polymers tend to lack immunogenicity regardless of their size. Copolymers of sufficient size, containing two or more different amino acids are immunogenic. The addition of aromatic amino acids, such as tyrosine or phenylalanine has immense effect on theimmunogenicity of these synthetic polymer. As for e.g. a synthetic copolymer of glutamic acid and lysine requires a minimum molecular wt. of 30,000-40,000 for immunogenicity. Besides chemical compositions, structural complexicity and heterogeneity of protein affect immunogenicity. Starting from nascent to final stage, proteins undergo four levels of organization called primary, secondary, tertiary and quarter- nary protein, which gradually add their structural complexity and impose effect on their immunogenicity

vi. Antigen processing and presentation:

There is a great variety of antigens found within the body. Some antigens are readily be recognized by immune system (mainly by B-cells) and some require to be processed and presented in a present able manner so that they can be recognized by immune cells (T-cells usually). There are intracellular(endogenous) and extracellular (exogenous) antigens which present different challenges to the immune system. A foreign protein (antigen) to be recognized by a T-cell must be degraded into small antigenic peptides that form physical complex with Class I or Class II Major Histo compatibility Complex(MHC) molecules. This conversion of proteins into MHC associated peptide fragments is called antigen processing and presentation. This processing of antigens is mediated by different antigen cells of the body

3.Functions of Antibodies

a.Agglutination

Agglutination is a process of clumping of particulate antigen with antibody. This is brought about by the linking of the particulate antigens by the two fab fragments of Immunoglobulin molecule.

b.Precipitation

Immunoglobulin combines with soluble antigens such as tetanus toxin and the complex thus formed becomes insoluble and precipitated. Thus, the toxin is made inactive.

c.Neutralization

The antibodies cover the toxic sites of the antigenic agents and thus neutral them (make them inactive).

d. Lysis

Some antibodies are capable of causing rupture of the cells. Ex-IgM

e.Opsonization

Opsonization is the process of coating the bacteria with antibodies (opsonins) and making the antigen more susceptible for phagocytosis. These antibodies are known as opsonizing antibodies. IgM performs this role very efficiently Some antibodies are capable of causing rupture of the cells. Eg. IgM Antibodies alter the surface properties of antigens such as the surface charge and thus may reduce the electrostatic repulsion between the phagocytes and antigens.The Fc fragments of the antibody have an affinity for the surface of the charge and thus may reduce the electrostatic repulsion between the phagocytesand antigens.

f.Tissue Fixation

The immunoglobulins attach to tissue cells and cause various hypersensitive (allergic) reactions. This tissue fixation is mainly carried out by the Fe portion of IgE. Ig E increases in conditions of hay fever and asthma and these antibodies bind firmlyto mast cells. Whenan appropriate antigen (allergen) enters the same person again, with the tissue fixed IgE antibody and a chain of events are triggered. Mast cells degranulate, Vasoactive amines are released.

g. Selective Transport

In human beings, IgG is transported from the mother to the foetus through the placenta. It gives passive immunity to the newborn baby. The new born is passively protected for about 6-9 months. IgA is also selectively transported and selectively secreted into the saliva,mucous secretions of the respiratory and digestive tracts and also into the colostrum.

4. HUMAN LEUKOCYTEANTIGEN

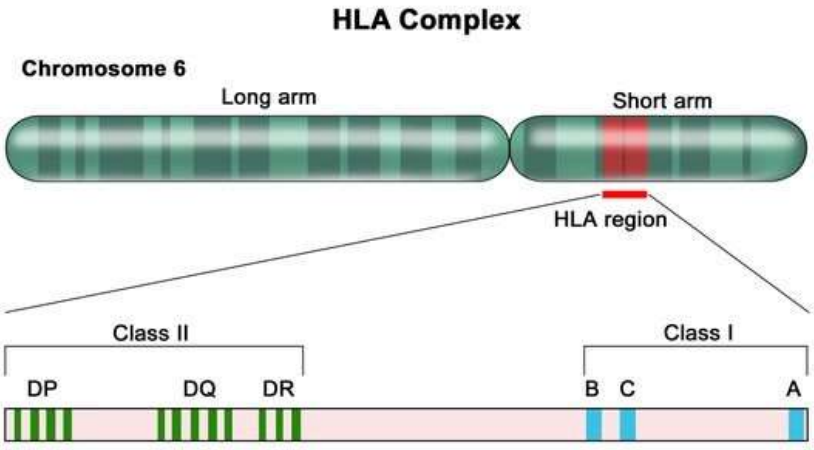
The human leukocyte antigen (HLA) system or complex is a complex of genes on chromosome6 in humans which encode cell-surface proteins responsible for the regulation of the immune system. The HLA system is also known as the human version of the major histocompatibility complex (MHC) found in many animals.

HLAs corresponding to MHC class I (A, B, and C), all of which are the HLA Class1 group, present peptides from inside the cell. For example, if the cell is infected by a virus, the HLA system brings fragments of the virus to the surface of the cell so that the cell can be destroyed by the immune system.

These peptides are produced from digested proteins that are broken down in the proteasomes. Foreign antigens presented by MHC class I attract T-lymphocytes called killer T-cells (also referred to as CD8-positive or cytotoxic T-cells) that destroy cells.

HLAs corresponding to MHC class II (DP, DM, DO, DQ, and DR) present antigens from outside of the cell to T-lymphocytes. These particular antigens stimulate the multiplication of T-helper cells (also called CD4-positive T cells), which in turn stimulate antibody-producing B-cells to produce antibodies to that specific antigen. Self-antigens are suppressed by regulatory T cells. Predicting which (fragments of) antigens will be presented to the immune system by a certain HLA type is difficult, but the technology involved is improving

HLAs corresponding to MHC class III encode components of the complement system.



5. ANAPHYLAXIS

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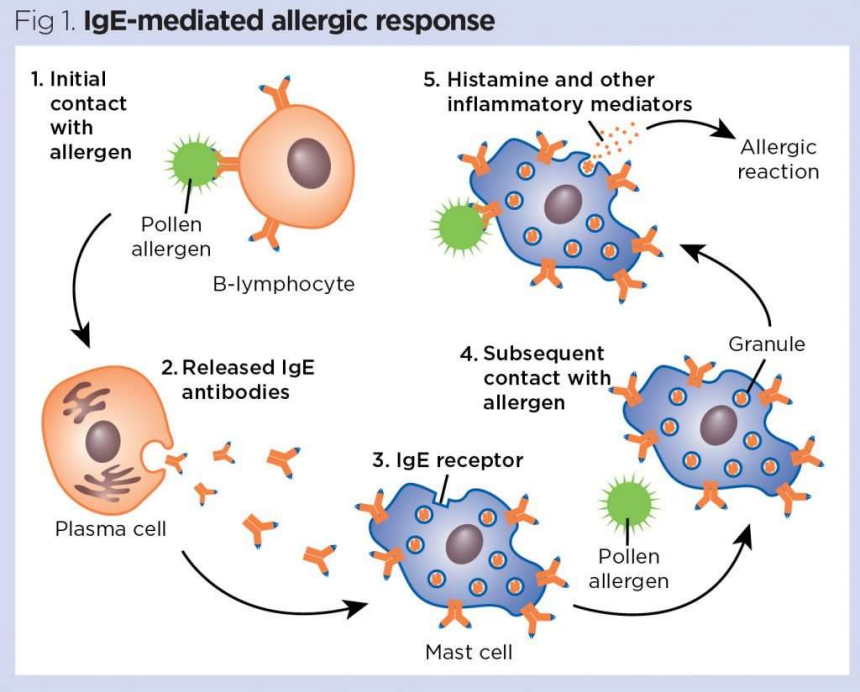


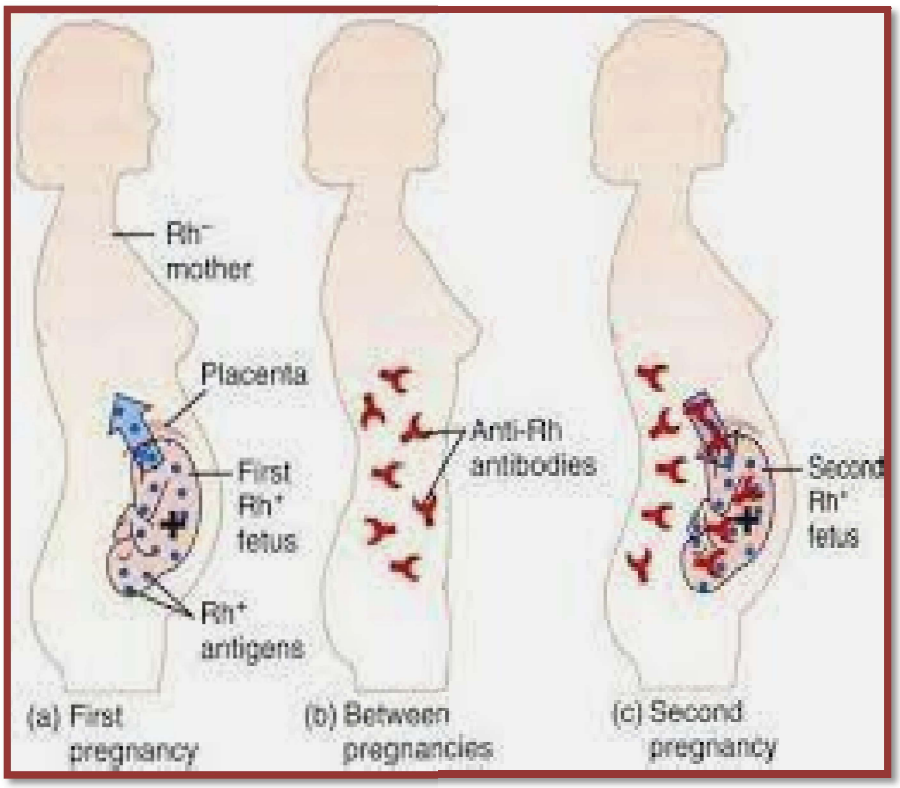
6. ERYTHROBLASTOSIS FOETALIS

Erythroblastosis foetalis is a haemolytic disease caused by the reaction of Rh antigen and Rh antibody. It occurs in the Rh+ baby developing in an Rh negative mother. The Rh antibody involved in this reaction belongs to IgG type. It is an isoimmunereaction.Landsteiner (1940) found an antigen in Rhesus monkey and it is called Rh antigen or antigen D. Some human beings possess this antigen on their RBC and others do not contain it.The persons containing antigen D on their RBC are called Rh positive.The persons who do not contain antigen D are called Rh negative. The Rh blood group system has no natural antibody. But when a person receives blood, either 1 ml or more, from a Rh+ person, the Rh- person responds and produces Rh antibody called

anti-D. The anti-D remains in his blood and it does not do any harm as possessor. Now he is sensitized to antigen D. When he receives the second time, antigen D and anti- D cross react with each other leading to haemolysis.

Such a type of haemolysis occurs in the Rh+ foetus and Rh negative lady. when an Rh negative lady marries an Rh +man, their foetus will be Rh+. The Rh+ baby develops in the uterus of the mother. The Rh+, baby contains antigen D. During delivery of this baby, the placentawith the rupture of some blood vessels connecting the foetus and the as a result some antigen D present in the blood of the baby mixeswith blood of the mother.

The blood of the Rh-negative mother responds and produces anti-D. The anti- D formed now persists in the blood throughout her life. The complication arises when she becomes pregnant for the second time. When the foetus makes contact with the mother through the placenta, anti-D passesfrom mothers'blood into the blood of the foetus. The anti-D combines with the Rh antigen present on the RBC of foetus. This binding causes the lysis of RBC of foetus. This haemolytic disease of the foetus is called Erythroblastosis foetalis. The lysis of RBC leads to jaundice and the baby is killed. All babies will meet the same consequences.



Now this problem can be solved by therapy. The negative mothers given a dose of anti-D, within 72 hours or soon after the first delivery. This step prevents sensitization of the mother.

UNIT – III

WRITE ABOUT VARIOUS CULTURE MEDIA USED FOR ANIMAL CELL CULTURE

Introduction

Cell culture is defined as the removal of cells, tissues or organs from an animal or plant and their subsequent placement into an artificial environment conducive to their survival and/or proliferation.

- Basic environmental requirements for cells to grow optimally are: controlled temperature, a substrate for cell attachment, and appropriate growth medium and incubator that maintains correct pH and osmolality.
- The most important and crucial step in cell culture is selecting appropriate growth medium for the in vitro cultivation.

A growth medium or culture medium is a liquid or gel designed to support the growth of microorganisms, cells, or small plants,comprise an appropriate source of energy and compounds which regulate the cell cycle.

- A typical culture medium is composed of a complement of amino acids, vitamins, inorganic salts, glucose, and serum as a source of growth factors, hormones, and attachment factors. In addition to nutrients, the medium also helps maintain pH and osmolality.

  Culture media are grouped into two categories

A) Natural media

B) Artificial media

Natural Media

Natural media consist only naturally occurring biological fluids. Natural media are very useful and convenient for a wide range of animal cell culture. The major disadvantage is poor reproducibility due to lack of knowledge of the exact composition of these natural media.

These are of three types –

1) Clots – commonly used clots are plasma clots, which are been used from long time.plasma is now available in liquid or lyophilized form. It may be prepared in laboratory usually from blood

2) Biological fluids- serum is most widely used biological fluid as culture media. Serum may be obtained from human blood, placental cord blood or horse blood. Serum is liquid excluded from coagulating blood. Different preparation of serum differ in their properties ,they have to be tested for their sterility and toxicity before use.

3) Tissue extracts- Chick embryo extract is the commonly used tissue extract. Other tissue extracts that are been used are liver, spleen, bone marrow .Tissue extracts can often be substituted by a mixture of amino acids and certain other organic compounds.

Artificial or Synthetic media

They are prepared by adding nutrients (both organic and inorganic), vitamins, salts, O_2 and CO_2 gas phases, serum proteins, carbohydrates, cofactors

Different artificial media have been devised to serve one or more of the following purposes:

1) Immediate survival (a balanced salt solution, with specific pH and osmotic pressure)

2) Prolonged survival (a balanced salt solution supplemented with various formulation of organic compounds and/or serum)

3) Indefinite growth

4) Specialized functions.

Artificial media are four types

1) Serum containing media

Fetal bovine serum is the most common supplement in animal cell culture media. It is used as a low-cost supplement to provide an optimal culture medium.

Advantages of serum in media	Disadvantages of serum in media
Serum contains various growth factors and hormones which stimulates cell growth and functions.	Lack of uniformity in the composition of serum
Helps in the attachment of cells	Testing needs to be done to maintain the quality of each batch before using
Acts as a spreading factor	May contain some of the growth inhibiting factors
Acts as a buffering agent which helps in maintaining the pH of the culture media	Increase the risk of contamination

Serum provides carriers or chelators for labile or water-insoluble nutrients, hormones and growth factors, protease inhibitors, and binds and neutralizes toxic moieties

2) Serum-free media

Presence of serum in the media has many drawbacks and can lead to serious misinterpretations in immunological studies . A number of serum-free media have been developed . These media are generally specifically formulated to support the culture of a single cell type,. These media are also referred to as 'defined culture media' since the components in these media are known.

3) Chemically defined media

These media contain contamination-free ultra pure inorganic and organic ingredients, and may also contain pure protein additives, like growth factors Their constituents are produced in bacteria or yeast by genetic engineering with the addition of vitamins, cholesterol, specific amino acids, and fatty acids

4) Protein-free media

Protein-free media do not contain any protein and only contain non-protein constituents. Compared to serum-supplemented media, use of protein-free media promotes superior cell growth and protein expression and facilitates downstream purification of any expressed product Formulations like MEM, RPMI-1640 are protein-free and protein supplement is provided when required.

DEFINE PRIMARY CELL CULTURE.EXPLAIN ANY TWO METHODS EMPLOYED FOR TISSUE DISAGGREGATION.

Introduction

Primary culture refers to the cells that are placed in culture directly from the tissue of origin. These are called primary cultures until the first subculture. After the first subculture, they may be called secondary cultures, and thereafter, if continued passage is possible, a cell line.

These cultures can contain mixed cell types or a single cell type. However, primary cultures are rare, if ever,consist of a single cell type. Attempts may be made to mechanically or enzymatically purify the cell type of interest during the tissue dissociation by culturing the cells in conditions inhibit or kill some major contaminant cell type(s).

The following criteria for efficient development of primary cultures-

- Mostly embryonic tissues are preferred for primary cultures than adult tissues. This is due to the fact that the embryonic cells can be

disaggregated easily and yield more viable cells, besides rapidly proliferating in vitro.

- The quantity of cells used in the primary culture should be higher since their survival rate is substantially lower (when compared to subcultures).

- The tissues should be processed with minimum damage to cells for use in primary culture. Further, the dead cells should be removed.

- Selection of an appropriate medium (preferably a nutrient rich one) is advisable. For the addition of serum, fetal bovine source is preferred rather than calf or horse serum.

- It is necessary to remove the enzymes used for disaggregation of cells by centrifugation.

Techniques for Primary Culture:

Mechanical Disaggregation:

For the disaggregation of soft tissues (e.g. spleen, brain, embryonic liver, soft tumors), mechanical technique is usually employed. This technique basically involves careful chopping or slicing of tissue into pieces and collection of spill out cells.The cells can be collected by two ways:

1) Pressing the tissue pieces through a series of sieves with a gradual reduction in the mesh size.

2) Forcing the tissue fragments through a syringe and needle.

Although mechanical disaggregation involves the risk of cell damage, the procedure is less expensive, quick and simple. This technique is particularly useful when the availability of the tissue is in plenty, and the efficiency of the yield is not very crucial. It must however, be noted that the viability of cells

obtained from mechanical techniques is much lower than the enzymatic technique.

Enzymatic Disaggregation:

Enzymatic disaggregation is mostly used when high recovery of cells is required from a tissue. Disaggregation of embryonic tissues is more efficient with higher yield of cells by use of enzymes. This is due to the presence of less fibrous connective tissue and extracellular matrix. Enzymatic disaggregation can be carried out by using trypsin, collagenase or some other enzymes.

Disaggregation by trypsin

The term trypsinization is commonly used for disaggregation of tissues by the enzyme, trypsin.

1) The crude trypsin is more effective due to the presence of other proteases
2) Cells can tolerate crude trypsin better.
3) The residual activity of crude trypsin can be easily neutralized by the serum of the culture media (when serum-free media are used, a trypsin inhibitor can be used for neutralization).

Disaggregation of cells can also be carried out by using pure trypsin which is less toxic and more specific in its action. The desired tissue is chopped to 2-3 mm pieces and then subjected to disaggregation by trypsin.

There are two techniques of trypsinization-warm trypsinization and cold trypsinization.

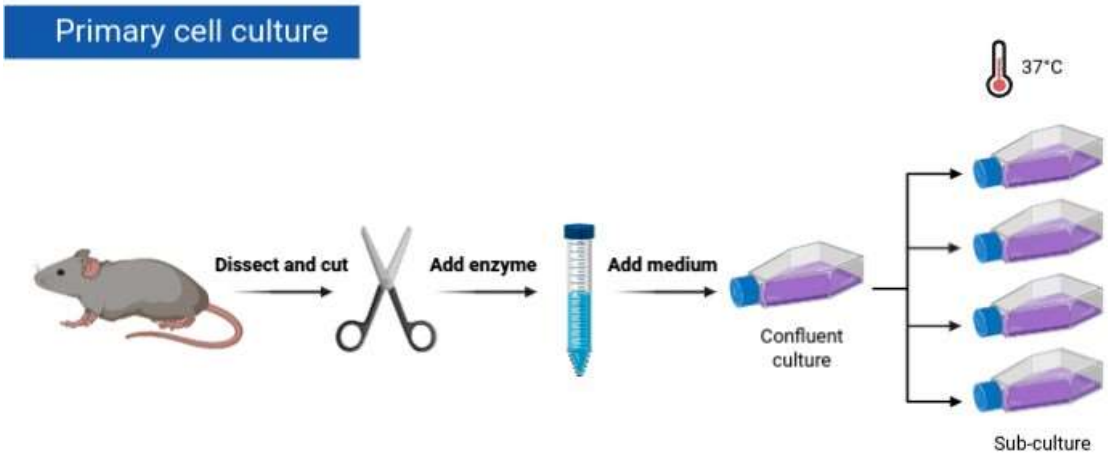
Warm trypsinization	Cold trypsinization
Warm trypsinization is the method of treating the cells with trypsin under warm conditions at a temperature of $36.5 - 37^{0}$.	Cold trypsinization is the process of trypsin treatment that takes place under colder conditions preferably in ice maintaining very low temperatures.
Lesser time required for the entire process (around 4 hours) of warm trypsinization.	Longer time required (around 6 – 24 hours) for cold trypsinization.
Yield of cells is low .	Yield of cells is high.
Centrifugation is required for disaggregation of cells.	Centrifugation is not required in cold trypsinization.
A larger quantity of tissue can be used.	A smaller quantity of tissue can be used .
Cell damage is high.	Cell damage is low.

Primary Explant Technique

The primary explant technique was developed by Harrison in 1907. This technique has undergone several modifications, and is still in use.
The tissue in basal salt solution is finely chopped, and washed by settlings. The basal salt solution is then removed. The tissue pieces are spread evenly over the growth surface. After addition of appropriate medium, incubation is carried out for 3-5 days. Then the medium is changed at weekly intervals until a substantial outgrowth of cells is observed. Now, the explants are removed and transferred to a fresh culture vessel.

The primary explant technique is particularly useful for disaggregation of small quantities of tissues (e.g. skin biopsies). The other two techniques mechanical or enzymatic disaggregation however, are not suitable for small amounts of tissues, as there is a risk of losing the cells.

The limitation of explant technique is the poor adhesiveness of certain tissues to the growth surface, and the selection of cells in the outgrowth. It is however, observed that the primary explant technique can be used for a majority of embryonic cells e.g. fibroblasts, myoblasts, epithelial cells,glial cells.



WRITE SHORT NOTES ON FOLLOWING

A) ORGAN CULTURE **B) CRYOPRESERVATION**

A) Organ culture

Organ culture can be defined as the organs or plant parts culturing in an artificial media or a culture from isolated medium. Any part of plant can serve as explants in organ culture-like shoot, root, leaf, and flower. Following are some techniques of organ culture:

1. Plasma clot method .

2. Agar gel techniques of organ culture

3. Organ culture in liquid media.

1.Plasma clot method

- Explant is placed and cultured on suitably prepared plasma clot kept in watch glass.
- Plasma clot is prepared by mixing 3 drops of chicken plasma and 1 drop of chick embryo extract (50 %) . One or two such watch glasses are kept in Petri dishes lined with cotton wool or moist filter paper to minimize evaporation of clot. May or may not be closed with lid and sealed with paraffin wax.
- Petri dish is then incubated at 37.5 0C. Fresh clots have been provided every 2 to 3 days for avian tissues and every 3 to 4 days for mammalian tissues.

Advantages:

Inexpensive

Permits light microscope hence suitable to study hair growth, fetal mouse skin differentiation

Disadvantages:

Clot liquefies near explants so explants can become immersed in the medium.Short duration of culture (Less than 4 weeks)

Biochemical analysis is not possible due to complexity of medium

Applications

To study action of hormones, vitamins, carcinogens on adult mammalian tissues.

To study morphogenesis in embryonic organ rudiments

2. Organ culture in Agar method

Problems faced by plasma clots is ended by using agar gels. It has been hosted by Spratt. Gaillard's techniques modified by Wolf and Heffner. In embryological watch glass agar gel is enclosed. This is successfully used for the developmental and morphogenetic studies. Although agar does not soften it is not examined or adapted without resettling into the culture. Fluid media over comes this difficulty while united by the provision which avoids the cultures being absorbed.

3. Organ Culture in Liquid Media

The liquid media consist of all the ingredients except agar. When liquid media are used for organ culture, generally perforated metal gauze or cellulose acetate or a raft of lens paper is used. These possibilities provide support.

Limitations

Results from organ cultures are often not comparable to those from whole animals studies, e.g. in studies on drug action since the drugs are metabolized in vivo but not in vitro.

B) Cryopreservation

Cryopreservation is a process that preserves organelles, cells, tissues, or any other biological constructs by cooling the samples to very low temperatures. The principle involved in cryopreservation is to bring the plant cell and tissue

cultures to zero metabolism or non-dividing state by mean of storage of germplasm at a very low temperatures,

(i) Over solid CO_2 (-79°C),
(ii) Deep freezers (-80°C),
(iii) in vapor phase nitrogen (-150°C),
(iv) in liquid nitrogen (-196°C).

Among these, the most commonly used is liquid nitrogen. At the temperature of liquid nitrogen (-196°C), almost all the metabolic activities of cells are ceased and the sample can then be preserved in such state for extended periods.

1. Collection and preservation of cells
2. Addition of cryoprotectants
3. Freezing method.
4. Storage
5. Thawing Techniques:

Rapid freezing method.
Slow freezing method.
Cryoprotective dehydration

1. Collection and preservation of cells

Biological materials,cell suspensions or thin tissue samples like Semen,Embryo (2,4 or 8 cell),Blood can be collected.In general, cryopreservation is easier for thin samples and small clumps of individual cells, because these can be cooled more quickly and so require lower doses of toxic cryoprotectants.

2. Addition of cryoprotectants
There are two potential sources of cell damage during cryopreservation.

1. Formation of large ice crystals inside the cell.
2. Intracellular concentration of solutes increase to toxic levels before or during freezing as a result of dehydration
Cryoprotectants acts like antifreeze,they lower freezing
temperature and increase viscosity.

3. Freezing method

A. Slow Freezing Method(SFM):-

The tissues is slowly frozen with decrease in temperature of -0.5°C to -5°C/ min from 0°c-100°c, and then transfer to liquid nitrogen.

B. Rapid Freezing Method(RFM):-

The material is plunged into liquid nitrogen decreases in temperature from -300°C to -1000°C/min or more, the quicker the freezing is done the smaller the ice is crystal.

C. Stepwise-Freezing Method:-
In this method slow freezing down to -20°C to -400°C ,a stop for a period of approximately 30 min and then additional rapid freezing to -196°C is done by plunging in liquid nitrogen.

4.Storage:

In general the frozen cells/tissues are kept for storage at a ranging from -70°C to -196°C. Storage is ideally done in liquid nitrogen refrigerator at -150°C in vapor phase or at -196°C in the liquid phase. The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability for long term. For long term storage temperature at -196°C in liquid nitrogen is ideal.

5. Thawing Techniques:

It is done by putting the ampoule containing the sample in a warm water (35°to 45°C)bath. By this approach, rapid thawing [at the rate of (500 to&750°C/min) occurs,this protects the cell from damaging effect of ice crystal formation. This transfer is necessary since the cells get damaged if left for long in warm (37°C - 45°C) water. For cryopreserved material(cells/tissues) where the water content has been reduced to an optimal level before freezing, the process of thawing become less critical.

AMPOULE

Benefits

It is useful in the breeding of dairy cattle.
The cryopreserved blood can stored for many years.
The cancer and tumour cells can be preserved for further research.
Stem cells, Umbilical cord blood , skin cells are preserved for further use.

Disadvantages

High cost and Social issues.

DISCUSS THE PRODUCTION OF MONOCLONAL ANTIBODIES AND ADD SHORT NOTES ON THEIR USES.

Hybridoma Technology

Introduction

Hybridomas are cells that can produce a desired antibody in large amounts, to produce monoclonal antibodies.

Monoclonal antibodies can be produced in specialized cells through a technique now popularly known as hybridoma technology.

Hybridoma technology was discovered in 1975 by two scientists, Georges Kohler of West Germany and Cesar Milstein of Argentina ,who jointly with Niels Jerne of Denmark),were awarded the 1984 Noble prize for physiology and medicine.

Production of Monoclonal Antibodies:

The establishment of hybridomas and production of MAbs involves the following steps

1) Immunization:

The very first step in hybridoma technology is to immunize an animal (usually a mouse), with appropriate antigen along with an adjuvant like Freund's complete or incomplete adjuvant.The injections at multiple sites are repeated several times. This enables increased stimulation of B-lymphocytes which are responding to the antigen.

Three days prior to killing of the animal, a final dose of antigen is intravenously administered. The immune-stimulated cells for synthesis of antibodies have grown maximally by this approach.

The concentration of the desired antibodies is observed in the serum of the animal at frequent intervals during the course of immunization.When the serum concentration of the antibodies is optimal, the animal is sacrificed.

The spleen is aseptically removed and disrupted by mechanical or enzymatic methods to release the cells. The lymphocytes of the spleen are separated from the rest of the cells by density gradient centrifugation.

2. Cell Fusion:

The thoroughly washed lymphocytes are mixed with HGPRT defective myeloma cells.

The mixture of cells is exposed to polyethylene glycol (PEG) for a short period,since it is toxic.

PEG is removed by washing and the cells are kept in a fresh medium.

These cells are composed of a mixture of hybridomas (fused cells), free myeloma cells and free lymphocytes.

3. Selection of Hybridomas:

When the cells are cultured in HAT (Hypoxanthine Aminopetrin Thymidine) medium. Aminopterin in the myeloma cells die, as they cannot produce nucleotides by the de novo or salvage medium blocks the pathway that allows for nucleotide synthesis.

Hence, unfused B cell dieas they have a short life span, only B cell-myeloma hybrids survive, since the HGPRT gene coming from the B cells is functional.

These cells produce antibodies (a property of B cells) and are immortal (a property of myeloma cells).Only the hybridoma cells grow, while the rest will slowly disappear. This happens in 7-10 days of culture.

Selection of a single antibody producing hybrid cells is very important. This is possible if the hybridomas are isolated and grown individually.

The suspension of hybridoma cells is so diluted that the individual sample contain on an average one cell each. These cells, when grown in a regular culture medium, produce the desired antibody.

4. Screening the Products:

The culture medium from each hybridoma culture is periodically tested for the desired antibody specificity. The two techniques namely ELISA and RIA are commonly used for this purpose.

In both the assays, the antibody binds to the specific antigen (usually coated to plastic plates) and the unbound antibody and other components of the medium can be washed off. Thus, the hybridoma cells producing the desired antibody can be identified by screening. The antibody secreted by the hybrid cells is referred to as monoclonal antibody.

5. Cloning and Propagation:

The single hybrid cells producing the desired antibody are isolated and cloned. Two techniques are commonly employed for cloning hybrid cellslimiting dilution method and soft agar method.

Limiting dilution method:

In this procedure, the suspension of hybridoma cells is serially diluted and the aliquots of each dilution are put into micro culture wells. The dilutions are so made that each aliquot in a well contains only a single hybrid cell. This ensures that the antibody produced is monoclonal.

Soft agar method:

In this technique, the hybridoma cells are cultured in soft agar. It is possible to simultaneously grow many cells in semisolid medium to form colonies. These colonies will be monoclonal in nature. In actual practice, both the above techniques are combined and used for maximal production of MAbs.

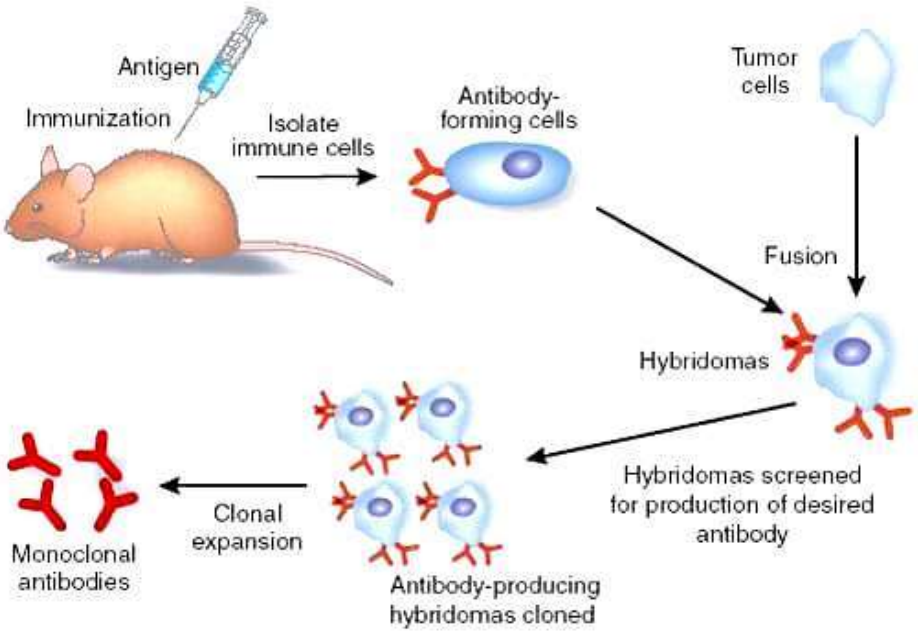
6. Characterization and Storage:

The monoclonal antibody has to be subjected to biochemical and biophysical characterization for the desired specificity.

It is also important to elucidate the MAb for the immunoglobulin class or sub-class, the epitope for which it is specific and the number of binding sites it possesses.

The stability of the cell lines and the MAbs are important. The cells (and MAbs) must be characterized for their ability to withstand freezing, and thawing.

The desired cell lines are frozen in liquid nitrogen at several stages of cloning and culture.



Applications of MAbs

- Monoclonal antibodies used as diagnostic reagents for biochemical analysis or as tools for diagnostic imaging of diseases.
- MAbsare used in the treatment of cancer, transplantation of bone marrow and organs, autoimmune diseases, cardiovascular diseases and infectious diseases.
- MAbs promote efficient opsonization of pathogenic organisms (by coating with antibody) and enhance phagocytosis.
- MAbs, against the antigens on the surface of cancer cells, are useful for the treatment of cancer. The antibodies bind to the cancer cells and destroy them.
- This is brought out by antibody—dependent cell-mediated cytotoxicity, complement-mediated cytotoxicity and phagocytosis of cancer cells (coated with MAbs) by reticuloendothelial system

GIVE A DETAILED ACCOUNT OF TYPES OF STEM CELLS AND THEIR USES.

Introduction

Stem cells are defined as cells that have clonogenic and self-renewing capabilities and differentiate into multiple cell lineages.

Stem cells are found from the early stages of human development to the end of life. Stem cells are basic cells of all multicellular organisms having the potency to differentiate into wide range of adult cells.Self renewal and totipotency are characteristic of stem cell.

A stem cell is a non-specialized, generic cell which can make exact copies of itself indefinitely and can differentiate and produce specialized cells for the various tissues of the body.

Stem cells are cells found in most, if not all, multi-cellular organisms. They are characterized by selfrenewal and potency i.e. - the ability to renew themselves through mitotic cell division and differentiating into a diverse range of specialized cell types.

They are vital to the development, growth, maintenance, and repair of our brains, bones, muscles, nerves, blood, skin, and other organs .

Laboratory studies of stem cells enable scientists to learn about the cells' essential properties and what makes them different from specialized cell types.

Stem cells have two important characteristics:

SELF RENEWAL

1. Obligatory asymmetric replication: – Each division produces, one daughter cell which is differentiated and Another cell which is identical to the parent cell

2.Stochastic differentiation: – When one stem cell develops into two differentiated daughter cell – Another stem cell undergoes mitosis and produces two daughter cell identical to the original.

POTENCY

It is the capacity to differentiate into specialized cell types,

Stem cells can be classified by the extent to which they can differentiate into different cell types. These four main classifications are totipotent, pluripotent, multipotent, or unipotent.

Totipotent- The ability to differentiate into all possible cell types.

Examples are the zygote formed at egg fertilization and the first few cells that result from the division of the zygote.

Pluripotent- The ability to differentiate into almost all cell types.

Examples include embryonic stem cells and cells that are derived from the mesoderm, endoderm, and ectoderm germ layers that are formed in the beginning stages of embryonic stem cell differentiation.

Multipotent -The ability to differentiate into a closely related family of cells. Examples include hematopoietic (adult) stem cells that can become red and white blood cells or platelets.

Oligopotent - The ability to differentiate into a few cells.

Examples include (adult) lymphoid or myeloid stem cells.

Unipotent - The ability to only produce cells of their own type, but have the property of selfrenewal required to be labeled a stem cell.

Examples include (adult) muscle stem cell

Classification of stem cells on the basis of their sources.

Early or embryonic and mature or adult. Early stem cells, often called embryonic stem cells, are found in the inner cell mass of a blastocyst after approximately five days of development.

Mature stem cells are found in specific mature body tissues as well as the umbilical cord and placenta after birth .

Embryonic stem cells- Embryonic stem cells are selfreplicating pluripotent cells that are potentially immortal. They are derived from embryos at a developmental stage before the time of implantation would normally occur in the uterus . The embryos from which human embryonic stem cells are derived are typically four or five days old and are a hollow microscopic ball of cells called the blastocyst .

Adult stem cells- Adult stem cells are undifferentiated totipotent or multipotent cells, found throughout the body after embryonic development, that multiply by cell division to replenish dying cells and regenerate damaged tissues.. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found.

Pluripotent stem cells- Recently, a third type of stem cell, with properties similar to embryonic stem cells, has emerged. Scientists have engineered these induced pluripotent stem cells (iPS cells) by manipulating the expression of certain genes - 'reprogramming' somatic cells back to a pluripotent state.

Applications

- Heart Disease- Adult bone marrow stem cells injected into the hearts are believed to improve cardiac function in victims of heart failure or heart attack
- Leukemia and Cancer - Studies show leukemia patients treated with stem cells emerge free of disease. -Injections of stem cells have also reduces pancreatic cancers in some patients. Proliferation of white cells
- Rheumatoid Arthritis -Adult Stem Cells may be helpful in jumpstarting repair of eroded cartilage.
- Parkinson's disease -Major motor features of disorder results from the loss of a single cell population i.e., dopaminergic neurons with in stratum nigra. This suggests that cell replacement should be adequate to treat it. .
- Type I Diabetes - Pancreatic cells do not produce insulin . Embryonic Stems Cells might be trained to become pancreatic islets cells needed to secrete insulin.

WRITE SHORT ON THE FOLLOWING

A) TYPES OF STEM CELLS B) HAT MEDIA

A) Types of stem cells

POTENCY It is the capacity to differentiate into specialized cell types

Classification of stem cells on the basis of potency

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Examples include (adult) muscle stem cell.

Classification of stem cells on the basis of their sources

The easiest way to categorize stem cells is by dividing them into two types:

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Pluripotent stem cells- Recently, a third type of stem cell, with properties similar to embryonic stem cells, has emerged. Scientists have engineered these induced pluripotent stem cells (iPS cells) by manipulating the expression of certain genes - reprogramming' somatic cells back to a pluripotent state.

B) HAT media

The HAT medium is one of the several strategies used for the selection of hybrid cells, and it is the most widely used. This medium is supplemented with **Hypoxanthine, Aminopterine, Thymidine**, hence the name HAT medium.

Anti metabolite **Aminopterine** blocks the cellular biosynthesis of purine and pyramidine from simple sugars and amino acids. However, normal human and mouse cells can still multiply as they can utilize hypoxanthine and thymidine present in the medium through the salvage pathway ,which ordinarily recycles the purines and pyramidine produced from degradation of nucleic acids .

Hypaxanthine is converted into Guanine by the enzyme **HGPRT** (hypoxanthine-guanine phoaphoribosyl transferase},

Thymidine is phosphorylated by thymidine kinase.

Both HGPRT and TK are the enzymes in the salvage pathway. On a Hat medium those cells that have active HGPRT and TK enzymes can proliferate, those with deficit in these enzymes cannot divide. For using HAT medium as a selective agent the human cells used for fusion must be deficit either for HGPRT enzyme or TK enzyme. While mouse cells must be deficient for other enzyme of that pair. Thus, one may fuse HGPRT deficient human cells with TK deficient mouse cells. Their fusion products will be TK + the human gene and HGPRT + and will multiply on the HAT medium. While the unfused human and mouse cells will fail to multiply as the above hybrid cell.

SHORT ANSWER QUESTIONS

CELL LINES

A cell line is a permanently established cell culture that will proliferate indefinitely in an appropriate fresh medium and space. Cell lines differ from cell

strains in that they do not follow the Hayflick limit and become immortalised. The Hayflick limit (or Hayflick Phenomenon) is the number of times a normal cell population will divide before it stops, presumably because the telomeres reach a critical length. A cell line arises from a primary culture at the time of the first successful subculture

Features of cell line:

- To use any cell line for the production of biological product, one should have knowledge of following things related to cell lines.
- Age, sex and species of the donor tissue.
- For human cell lines, the donor's medical history and if available, the results of tests performed on the donor for the detection of adventitious agents.
- Culture history of the cell line including methods used for the isolation of the tissues from which the line was derived, passage history, media used and history of passage in animals, etc.
- Previous identity testing and the results of all available adventitious agents testing.

Characteristics of Cell lines:

- The growth pattern and morphological appearance of the cell line should be determined and should be stable from the master cell bank to the end-of-production cells.
- If there are specific markers that may be useful in characterizing the cell line (such as marker chromosomes, specific surface markers), these should be characterized for stability.
- Mostly cultured cell lines are allowed to generate their own ECM (extra cellular matrix), but primary culture and propagation of some specialized cells, exogenous provision of ECM.
- Many transformed cell lines have provided the best model for the induction of differentiation.
- Since normal cells has limited dividing capacity, therefore after a fixed number of population doublings cell lines derived from normal tissue will die out. This is a genetically determined event involving several different genes and this phenomenon is known as senescence.

- If the cells have an identified finite life expectancy, the total number of population doubling levels through senescence should be determined.

Requirement of cell lines:

For the maintenance of Cell line some basic conditions are required. These are described as follows.

pH: Most cell lines grow well at p^H 7.4

Buffering: Culture media must be buffered under two sets of conditions:

a) Open dishes, where the evolution of CO2 causes the pH to rise

b) Overproduction of CO2 and lactic acid in transformed cell lines at high cell concentrations, when the pH will fall

Temperature: The temperature recommended for most human and warm-blooded animal cell lines is 37°C, closely to body heat, but generally set a little lower for safety.

Applications:

The generation of stably-transfected cell lines is essential for a wide range of applications:

Cell line can be used for gene function studies

Drug discovery assays or the production of recombinant proteins can be carried out by cell lines.

In contrast to transient expression, stable expression of cell line allows long term,as well as defined and reproducible expression of the gene of interest.

SOMATIC CELL FUSION

Somatic cells of different types can be fused to get hybrid cells. Hybrid cells are useful in variety of ways like-

- To study the control of cell division and gene expression.
- To investigate malignant transformations.

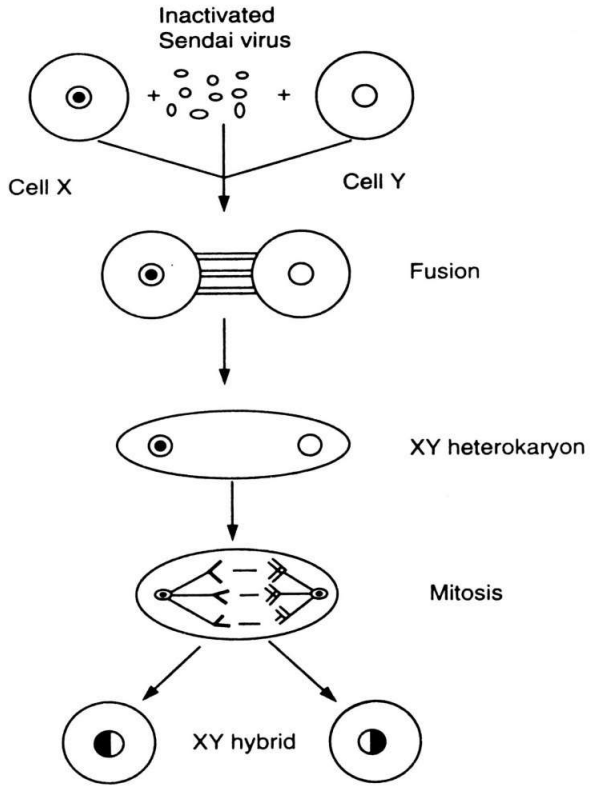
- For gene or chromosome mapping.
- Production of monoclonal antibodies.
- To obtain viral replication.

Generally human fibrocytes or leucocytes are fused with mouse cell lines. When human and mouse cells are mixed spontaneous cell fusion occurs at a very low rate. Cell fusion is enhanced 100 to 1000 times by the addition of ultraviolet inactivation sednai virus or a chemical called Polyethylne glycol. These agents adhere to the plasma membrane of the cells and alter their properties in such a waythat facilitates their fusion.

Fusion of two cells produce a single cell with in two nuclei,one from one of the cell entering fusion. Such a cell is called heterokaryon.

Subsequently the two nuclei also fuse to yield hybrid cell with a single nucleus.

A generalized scene for somatic cell hybridization may be described as follows.



Appropriate human and mouse cells are selected and mixed together in the presence of Sendai virus or PEG to promote cell fusion. After the fusion treatment ,the cells are washed ,suspended in complete medium, incubated overnight and then are plated on a selective medium , ex.,HAT medium which allow the

multiplication of hybrid cells are thus isolated and subjected to both cytogenetic and appropriate biochemical analysis for the detection of enzyme /protein/trait under investigation.

ESTABLISHED CELL LINES

Established Cell line is a cell strain is derived either from a primary culture or a cell line by either selection or cloning of cells having a specific properties.Cell strains are cells that have been adapted to culture but unlike cell lines, have a finite division potential.

Ex: (1) MRC (2) Hela, (3) CHO, (4) BHK, (5)Vero etc.

1. MRC :

MRC cell line means Medical Reserach Council cell strain. It is a diploid human cell culture line composed of fibroblasts derived from lung tissue of a 14 week old aborted caucasian male fetus.

- The cell line was isolated by J.P. Jacobs
- MRC cell lines are used to produce several vaccines including Measles, Mumps and Rubella (MMR), varicella and polio causing viral organisms.
- Infected MRC cells secrete the virus and can be cultured in large volumes suitable for commercial production.

2. Hela Cell lines:

Hela is a cell type in an immortal cell line used in scientific research.

It is the oldest & most commonly used Human cell line.

The cell line was derived from cervical cancer patient,Henrietta Lacks,who died of cancer.

The cell lines are remarkably durable and use in scientific research.

Uses of Hela Cell lines

- Stable growth of Hela enabled to successfully grow polio virus, facilitating the development of a vaccine.
- Hela cells were the 1st human cells successfully cloned and demand for the

- Hela cells have been used to test human sensitivity to tape, glue, cosmotics and many other products.

3. CHO Cell line:

Chinese hamster Ovary CHO Cell line consists of epithelial cell lines derived

from the ovary of the chinese hamster. This was first developed in 1957.

Uses :

- CHO cell line is used in Biological & medical research and commercially in the production of therapeutic proteins.
- CHO cell line has wide use in the studies of genetics, toxicity screening, nutrition and gene expression, particularly to express recombinant proteins.
- CHO cell line is a good model for radiation cytogenetics and tissue culture.

4. BHK Cell Lines:

- Baby Hamester Kidney Cell line (BHK) having fibroblasts was derived in 1961 by 1.A. Macpherson and M.G.P. Stoker and was used for first time in Molecular Biology.
- BHK cell lines are usefull for transformation and for stable and temporary transfection.
- BHK cells are susceptible to Human Adenovirus D, Reovirus 3, and Vesicular stomatitis virus.
- BHK cells are resistent to poliovirus 2 and Rabbit vesicular virus.
- BHK cells are negative for reverse transcriptase which means they lack genomes for integral retrovirus.

5. Vero Cell lines:

The Vero cel line was isolated from kidney epithelial cells extracted from an African green Monkey.Japan (Vero = green kidney).

These cell lines are used :

(a) in screening for toxin of E.Coli.

(b) as host cell for eukaryotic parasites specially of the trypanosomatids.

(c) as host for growing viruses.

UNIT – IV

LONG ANSWER QUESTIONS

WRITE AN ESSAY ON RESTRICTION ENDONUCLEASES.

The nuclease enzyme that cuts the DNA at a unique sequence is called restriction enzymes.They cut the DNA in a non terminal region. There are two different kinds of restriction enzymes:

1) Exonucleases

Catalyses hydrolysis of terminal nucleotides from the end of DNA or RNA molecule either 5'to 3' direction or 3' to 5' direction.

Example: exonuclease I, exonuclease II etc.

2) Endonucleases

It can recognize specific base sequence (restriction site) within DNA or RNA molecule and cleave internal phosphodiester bonds within a DNA molecule. **Example:** EcoRI, Hind III, BamHI etc

Restriction endonucleases are used to generate re-joinable DNA fragments.

1. They are also known as molecular knives, molecular scissors, restriction enzymes or molecular scalpels.

2. The sequence recognised by the restriction enzyme to cut the DNA is called Restriction site, restriction endonuclease site, recognition site. The recognition site consists of 4-8 box pairs.

3. The enzyme breaks two phosphodiester bonds, one in either strand of the duplex DNA to cut the DNA. The 3 cut end has a free - OH group and the 5' cut end have a phosphate group.

4. Some restriction enzymes recognise palindromic sequences to cut DNAs, but some others recognise non palindromic sequences.

5. The genome of an organism has several restriction sites for one restriction enzyme. The distance between two adjacent restriction sites varies greatly. So a restriction enzyme produces several DNA fragments of different length while cutting the DNA

Types of Restriction enzymes:

The restriction endonucleases are grouped into 4 types. They are:

i. Type-I restriction endonucleases

ii. Type-II restriction endonucleases

iii. Type-III restriction endonucleases

iv. Type IV restriction endonucleases

The type-I and type-III restriction enzymes recognize specific sequences in the duplex DNA but cut out the far away from the recognition sites. So they are not useful for genetic engineering.

Type I Enzymes

Type I enzymes are complex, multisubunit, combination restriction-and-modification enzymes that cut DNA at random far from their recognition sequences.

Type I enzymes are of considerable biochemical interest, but they have little practical value since they do not produce discrete restriction fragments or distinct gel-banding patterns.

These enzymes are bi/multifunctional and are capable of both restriction digestion and modification activities.

The cofactors S-Adenosyl methionine, hydrolyzed adenosine triphosphate (ATP), andmagnesium (Mg^{2+}) ions, are required for their full activity

E.g.- Eco RI, Hind III etc.

Type II Restriction Endonucleases:

A type-II restriction endonuclease recognises a specific sequence in the duplex DNA and cuts the DNA at the recognised sequence. So the cutting is sequence specific.

The enzyme consists of two identical sub-units and its molecular weight ranges from 20,000 to 100,000 daltons.

It requires Mg2+ as cofactor for the enzyme activity:

Eco RI. Hind III, MboI, etc. are examples for type II restriction enzymes.

At present, about 350 type II restriction endonucleases are isolated from various bacterial strains.

Type III Restriction Enzymes

Type III enzymes are also large combination restriction-and-modification enzymes. They cleave outside of their recognition sequences and require two such sequences in opposite orientations within the same DNA molecule to accomplish cleavage,they rarely give complete digests.

Bifunctional enzyme and heterodymer having more than one sub unit.Big subunit becomes functional in the absence of small unit. DNA is not cleaved within this molecule.

It is cut about 20-30 base pairs after the recognitionsite.Occasionally used in R-DNA technology.

DNA cleavage is aided by ATP as well as Mg^{2+} whereas SAM (S-Adenosyl Methionine) is responsible for stimulating cleavage.

Type IV Enzymes

Type IV enzymes recognize modified, typically methylated DNA and are exemplified by the McrBC and Mrr systems of E. coli.

Requires Mg^{2+} for it's functional activity.

It has one catalytic site per strand.It acts as heterodymer.

No subunit works in the absence of other. Cleaves modified or methylated DNA.

MODE OF ACTION

Restriction enzymes recognize a specific sequence of nucleotides called **"Recognition Site"** and produce a double-stranded cut in the DNA,called **"Restriction Site".** The recognition sequences can also be classified by the number of bases in its recognition site.

The inverted repeat palindrome is also a sequence that reads the same forward and backward,but the forward and backward sequences are found in complementary DNA strands (i.e., of double-stranded DNA), as in GTATAC (GTATAC being complementary to CATATG)

The ends in this case refer to the ends of the DNA strands. Depending on where and how the restriction enzyme cuts, it will produce either **Sticky ends or Blunt ends.**

Sticky ends / Cohesive ends get their name because they have overlaps that allow the two ends to base-pair and join together with another DNA.

5' G|A A T T C **3'**-->>Staggered Cut

3' C T T A A|G **5'**

Blunt ends / Non-Cohesive ends have no overlap

5' G A A|T T C **3'** -->> Straight Cut

3' C T T |A A G **5'**

NOMENCLATURE

Restriction enzymes are named according to the micro-organism in which they were discovered.The restriction enzyme **'HindIII'**, for example, is the third of several endonuclease activities found in the bacterium Haemophilus influenzae serotype d. The prefix 'R.' is added sometimes to distinguish restriction enzymes

from the modification enzymes with which they partner in vivo. Thus, 'R.HindIII' refers specifically to the restriction enzyme, and 'M.HindIII' to the modification enzyme. When there is no ambiguity, the prefix 'R.' is omitted.

EcoRI ,a common restriction enzyme,is named after E.coli ,strain R,isolate I since it was the first restriction enzyme isolated from strain R of E. coli

USES

1. Restriction enzymes are used to cut a source DNA into small fragments for the isolation of a desired gene to be cloned.

2. They are used to cut out unwanted sequences from natural Vector DNAs to construct active vectors.

3. They are used to cut the vector DNAs, at well defined sites for cloning purpose.

4. They are used to cut a large DNA into small fragments for nucleotide sequencing.

5. They are used to construct Restriction map of DNAs.

6. They are used to determine variants of closely related individuals by restriction fragment length polymorphism (RELP).

DESCRIBE ANY THREE TYPES OF ENZYMES USED IN R-DNA TECHNOLOGY.

A) Terminal deoxynucleotidyl Transferase(TdT) or DNA Nucleotidyl Transferase or Terminal Transferases

Terminal Deoxynucleotidyl Transferase (TdT), is a template-independent DNA polymerase, catalyzes the repetitive addition of deoxyribonucleotides to the 3'-OH of oligodeoxyribonucleotides and single- stranded and double-stranded DNA .

It is a specialized DNA polymerase expressed in immature, pre-B, preT lymphoid cells, and acute lymphoblastic leukemia/lymphoma cells.

In humans, terminal transferase is encoded by the DNTT gene.

Structure

Monomer

Molecular weight 58,000 Da.

In Mammals it shows two isomers

TdTS - Short form with 509 amino acids

TdTL – Long form with 529 amino acids

It shows 3'-5' exonuclease activity.

Function and Regulation

Catalyses addition of nucleotides to 3'-terminal end of the DNA.It does not require template.

It can add nucleotides to the blunt ends and sticky ends.

Cobalt is a necessary Co-factor

It's activity is inhibited by ammonium,chloride and phosphate anions.

Polymerization require divalent cations in the order of efficiency

Mg>Zn>Co>Mn

TdT perform nucleotide elongation during V (D) J recombination

V - Variable

D - Diversity

J – Joining

In acquired or adaptive immunity, T and B cells are mainly found.

When antigen enters into the body,they are produced in more number and give protective response.

Over the period of time,pathogen specific T-cells, undergo apoptosis,but some are resides in lymphoid and non-lymphoid tissues.

The immunological memory displayed by adaptive immune system provides longlasting protection against infection.

At molecular level,the cells of immune system increases acquired immunity against subsequent biological infections.

This is called V(D)J recombination, it requires certain enzymes activity,nucleases,polymerases and ligases.

TdT plays main role in this process.

TdT incorporate nucleotides which gives rise to generating immunoglobulins

Isoforms of TdT in humans :

TdTs :

In humans,short TdT (hTdTS) performing nucleotide elongation.If expression is beyond its limits it reduce efficiency of V (D) short TdT performing nucleotide elongation.If expression is beyond its limits it reduce efficiency of V(D)J.

Long TdT in humans (TdTL) can exist in two forms,they are hTdTL1 and hTdTL2.

hTdTS or hTdTL2 independently reduce efficiency of V(D)J recombination hTdTS and hTdTL2 restore recombination frequency back to normal.

hTdTS ,hTdTL1 and hTdTL2 can destroy recombination frequency.

Applications :

- Terminal transferase has applications in molecular biology. It can be used in RACE to add nucleotides that can then be used as a template for a primer in subsequent PCR.
- It can also be used to add nucleotides labeled with radioactive isotopes for example in the TUNEL assay(Terminal deoxynucleotidyl transferase

dUTP Nick End Labeling) for the demonstration of apoptosis (which is marked, in part, by fragmented DNA).

- It is also used in the immunofluorescence assay for the diagnosis of acute lymphoblastic leukemia.
- In immunohistochemistry and flow cytometry, antibodies to TdT can be used to demonstrate the presence of immature T and B cells and pluripotent hematopoietic stem cells, which possess the antigen.

B) DNA Polymerases:

DNA polymerases are enzymes that catalyse the

• synthesis of a new DNA strand from a pre existing strand. The enzyme adds de-oxyribonucleotides to the free 3'oH of the chain undergoing elongation the direction of synthesis is 5'- 3' for this activity. It requires (1) A template strand.

- A primer with a free 3'-oH (3) a pool of four dvTPs that synthase new strand of DNA.
  There are different types of DNA polymerases used in recombinant DNA technology.
  They are

1. E. Coli DNA Polymerase I

2. Klenow fragment

3. Thermo stable DNA polymerase

4. Reverse transcriptase

- E. Coli DNA polymerase I enzymes has both DNApolymerase as well as DNA nuclease activity.
- The klenow fragment can synthesize new DNA strand¬ complementary to the template but cannot degrade the existing strand
- Thermo stable DNA polymerases are a class of DNA¬ polymerase that remain functional even at high temperature. They are isolate from

Thermus aquaticus bacteria that live in hot springs. Also called taqpolymerase mainly used in PCR technique.

- Reverse transcriptase is a RNA dependent DNA¬ polymerase found in RNA viruses also called Retroviruses. It is involved in replication of Retroviruses.

Eukaryotic DNA Polymerases

Polymerase Types	Function
α	Initiates DNA replication in conjunction with primase
ε	Replication of the leading strand during S-phase
δ	Replication of the lagging strand during S phase
λ	Replication of mitochondrial DNA
η, κ, ι, ξ	Replication of damaged DNA

Prokaryotic DNA Polymerases:

5 types of DNA polymerases are found in E. coli

DNA polymerase I: functions in repair and replication

DNA polymerase II: functions in DNA repair

DNA polymerase III: main DNA replication enzyme

DNA polymerase IV: functions in DNA repair

DNA polymerase V: functions in DNA repair

C)Phosphatases:

• Phosphatase catalyses the cleavage of a phosphate $[PO4]^{2-}$ group from substrate by using a water molecule (hydrolytic cleavage).

• This reaction is not reversible.

• This shows totally opposite activity from enzyme like kinase and phosphorylase that add a phosphate group to their substrate.

On the basis of their activity there are two types of phosphatase they are

1.Acid phosphatase and

2.Alkaline phosphatase.

In both forms the alkaline phosphatase are most common.

• Special class of phosphatase that remove a phosphate group from protein, called "Phosphoprotein phosphatase".

Acid phosphatase :

• It shows its optimal activity at pH between 3 and 6, e.g. a lysosomal enzyme that hydrolyze organic phosphates liberating one or more phosphate groups. They are found in prostatic epithelial cells, erythrocyte, prostatic tissue, spleen, kidney etc.

Alkaline phosphatase:

• Alkaline phosphatase was the first zinc enzyme discovered having three closed spaced metal ion. Two Zn+2 ions and one Mg+2 ion, in which Zn+2 ions are bridges by Asp 51.

• In human body it is present in four isoforms, in which three are tissue specific isoform i.e. placental, germ cell, intestinal and one is non tissue specific isoform. The genes that encode for tissue specific isoforms are present on chromosome 2, while the genes for one non tissue specific are present on chromosome 1

• During post-translational modification, alkaline phosphatase is modified by N-glycosylation. It undergoes a modification through which uptake of two Zn+2 ion and one Mg+2 ion occurs which is important in forming active site of that enzyme. Alkaline phosphatases are isolated from microorganisms, tissue of different organs, connective tissue of invertebrate and vertebrate, and human body.

Placental Alkaline Phosphatase

The human placental ALP gene was mapped to chromosome 2. Placental ALP is a polymorphic enzyme, with up to 18 allozymes resulting from point mutations, in contrast to the other ALP isoenzymes.

Bacterial Alkaline Phosphatase (BAP)

Bacterial alkaline phosphate is a phosphor monoester that hydrolyzes 3' and 5' phosphate from nucleic acid (DNA/ RNA). It more suitably removes phosphate group before end labeling and remove phosphate from vector prior to insert ligation. BAP generally shows optimum activity at temperature 65°C. BAP is sensitive to inorganic phosphate so in presence of inorganic phosphates activity may reduce.

Calf Intestinal Alkaline Phosphatase (CIP)

It is isolated from calf intestine, which catalyzes the removal of phosphate group from 5' end of DNA as well as RNA. This enzyme is highly used in gene cloning experiments, as to make a construct that could not undergo self-ligation. Hence after the treatment with CIP, without having a phosphate group at 5' ends a vector cannot self ligate and recircularise. This step improves the efficiency of vector containing desired insert.

Shrimp Alkaline Phosphatase (SAP)

Shrimp alkaline phosphatase is highly specific, heat labile phosphatase enzyme isolated from arctic shrimp (Pandalus borealis). It removes 5' phosphate group from DNA, RNA, dNTPs and proteins. SAP can be irreversibly inactivated by heat treatment at 65°C for 15mins. SAP is used for 5' dephosphorylation during cloning experiments for various application as follows:

Dephosphorylate 5'-phosphate group of DNA/RNA for subsequent labeling of the ends To prevent self-ligation of the linearized plasmid.

To prepare PCR product for sequencing.

To inactivate remaining dNTPs from PCR product.

Two primary uses for alkaline phosphatase in DNA modification:

- Removing 5' phosphate from different vector like plasmid, bacteriophage after treating with restriction enzyme. This treatment prevents self ligation .
- Another important use of alkaline phosphatase is as a label for enzyme immunoassays.

D) DNALigases

DNA ligases are the enzymes that **catalyses the formation of phosphodiester bond between the deoxynucleotide** residues of two DNA strands and plays an essential role in maintaining genomic integrity by joining breaks in the phosphodiester backbone of DNA that occur during replication and recombination.Three human genes, LIG1, LIG3 and LIG4 encode ATP-dependent DNA ligases.

The LIG3 gene is less widely distributed.

Protein Partners of DNA Ligase III

XRCC1 (X-ray Repair Cross-Complementing Protein)

PARP1 (Poly [ADP-ribose] polymerase 1)

TDP1 (Tyrosyl phosphodiesterase 1)

NEIL1 and **NEIL2**

TYPES OF DNA LIGASES

1)Mammalian DNA Ligase:

In mammals, there are four specific types of ligase.

- **DNA ligase I:** Ligates the nascent DNA of the lagging strand after the Ribonuclease H has removed the RNA primer from the Okazaki fragments.
- **DNA ligase III:** Complexes with DNA repair protein XRCC1 to aid in sealing DNA during the process of nucleotide excision repair and recombinant fragments. Of the all known mammalian DNA ligases, only Lig III has been found to be present in mitochondria.
- **DNA ligase IV:** Complexes with XRCC4. It catalyzes the final step in the nonhomologous end joining DNA double-strand break repair pathway. It is also required for V(D)J recombination.
- **DNA ligase II:** A purification resulting from proteolytic degradation of DNA ligase III. Initially, it has been recognized as another DNA ligase and it is the reason for the unusual nomenclature of DNA ligases.

DNA ligase from eukaryotes and some microbes uses Adenosine Triphosphate (ATP) rather than NAD.

2)T4 Ligase:

The T4 ligase is the most-commonly used in laboratory research.It can ligate either cohesive or blunt ends of DNA, oligonucleotides but not single-stranded nucleic acids. It can also ligate blunt-ended DNA with much greater efficiency than E. coli DNA ligase. T4 DNA ligase cannot utilize NAD and it has an absolute requirement for ATP as a cofactor.

3)E.Coli: The E. coli DNA ligase is encoded by the lig gene. DNA ligase in E. coli, as well as most prokaryotes, uses energy gained by cleaving NAD to create the phosphodiester bond.

Mechanism of Action of DNA Ligases:

• ATP, or NAD+, reacts with the ligase enzyme to form a covalent enzyme–AMP complex in which the AMP is linked to ε -amino group of a lysine residue in the active site of the enzyme through a phosphoamide bond.

• The AMP activates the phosphate group at the 5´-end of the DNA molecule to be joined. It is called as the donor.

• The final step is a nucleophilic attack by the 3′-hydroxyl group on this activated phosphorus atom which acts as the acceptor. A phosphodiester bond is formed and AMP is released.

• The reaction is driven by the hydrolysis of the pyrophosphate released during the formation of the enzyme–adenylate complex. Two high-energy phosphate bonds are spent in forming a phosphodiester bond in the DNA backbone with ATP serving as energy source.

• The temperature optimum for T4 DNA ligase mediated ligation in vitro is 16°C. However ligation is also achieved by incubation at 4°C by incubating over night or at room temperature condition by incubating for 30 minutes.

• Adenylate and DNA-adenylate are the important intermediates of the phosphodiester bond forming pathway.

Applications:

DNA ligase enzyme is used by cells to join the "okazaki fragments" during DNA replication process. In molecular cloning, ligase enzyme has been routinely used to construct a recombinant DNA. Ligase enzyme used in molecular cloning.Joining of adapters and linkers to blunt end DNA molecule.

Cloning of restricted DNA to vector to construct recombinant vector.

DISCUSS ANY TWO GENE TRANSFER TECHNIQUES.

Gene transfer or uptake of DNA refers to the process that moves a specific piece of DNA into cell. The directed desirable gene transfer from one organism to another and the subsequent stable integration & expression of foreign gene into the genome is referred as genetic transformation. The transferred gene is known as transgene and the organism that develop after a successful gene transfer is known as transgenic.
Genetic engineering techniques and tools are artificial means of creation of variation. These can be used in development of new genotypes or species.

PHYSICAL OR MECHANICAL METHODS:-

Various physical or mechanical methods are employed to overcome this and aid

In gene transfer as listed below-

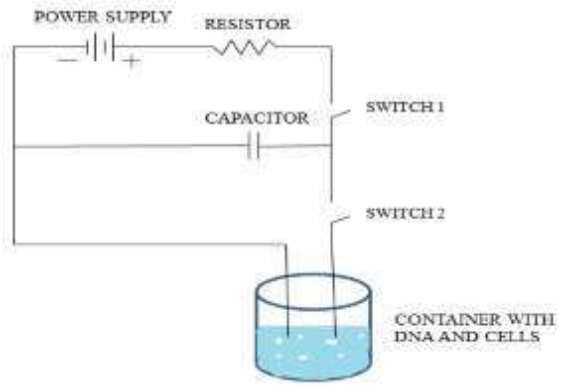
a)Electroporation

Electroporation is a mechanical method used for the introduction of polar molecules into a host cell through the cell membrane. It involves use of a large electric pulse that temporarily disturbs the phospholipid bilayer, allowing the passage of molecules such as DNA.
The basis of electroporation is the relatively weak hydrophobic/hydrophilic interaction of the phospholipids bilayer and ability to spontaneously reassemble after disturbance. A quick voltage shock may cause the temporary disruption of areas of the membrane and allow the passage of polar molecules. The membrane reseals leaving the cell intact soon afterwards.

Principle:

The phospholipid molecules of the plasma membrane are not static. When we apply electric field to them their kinetic energy increases resulting in the increase in the membrane permeability at certain points. This is exactly where we see the formation ofelectro-pores. The recombinant DNA can pass through these transient pores before they close.

Procedure: The host cells and the DNA molecules to be transported into the cells are suspended in a solution.



- When the first switch is closed, the capacitor charges up and stores a high voltage which gets discharged on closing the second switch.
- Typically, 10,000-1,00,000 V/cm in a pulse lasting a few microseconds to a millisecond is essential for electroporation which varies with the cell size.

- This electric pulse disrupts the phospholipid bilayer of the membrane causing the formation of temporary aqueous pores.
- When the electric potential across the cell membrane is increased by about 0.5-1.0 V, the charged molecules e.g. DNA migrate across the membrane through the pores in a similar manner to electrophoresis.
- The initiation of electroporation generally occurs when the transmembrane voltage reaches at 0.5-1.5 V. The cell membrane discharges with the subsequent flow of the charged ions and molecules and the pores of the membrane quickly close reassembling the phospholipid bilayer.

Advantages

- It is highly versatile and effective for nearly all cell types and species.
- It is highly efficient method as majority of cells take in the target DNA molecule.
- It can be performed at a small scale and only a small amount of DNA is required as compared to other methods.

Disadvantages

- Cell damage is one of the limitations of this method caused by irregular intensity pulses resulting in too large pores which fail to close after membrane discharge.
- Another limitation is the non-specific transport which may result in an ion imbalance causing improper cell function and cell death.

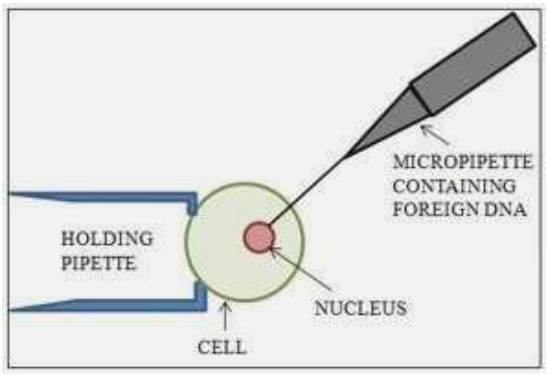
b)Microinjection

- DNA microinjection was first proposed by Dr. Marshall A. Barber in the early of nineteenth century.
- This method is widely used for gene transfection in mammals.
- It involves delivery of foreign DNA into a living cell (e.g. a cell, egg, oocyte, embryos of animals) through a fine glass micropipette. The introduced DNA may lead to the over or under expression of certain genes.
- It is used to identify the characteristic function of dominant genes.

Procedure

- The delivery of foreign DNA is done under a powerful microscope using a glass micropipette tip of 0.5 mm diameter.

- Cells to be microinjected are placed in a container. A holding pipette is placed in the field of view of the microscope that sucks and holds a target cell at the tip. The tip of micropipette is injected through the membrane of the cell to deliver the contents of the needle into the cytoplasm and then the empty needle is taken out.



Advantages

- No requirement of a marker gene.
- Introduction of the target gene directly into a single cell.
- Easy identification of transformed cells upon injection of dye along with the DNA.
- No requirement of selection of the transformed cells using antibiotic resistance or herbicide resistance markers.
- It can be used for creating transgenic organisms, particularly mammals.

Disadvantages

- Only one cell receives DNA per injection
- Handling of protoplasts for microinjection requires skilled persons
- Sophisticated equipment is required for this method
- Requirement of regeneration process from microinjected cells.

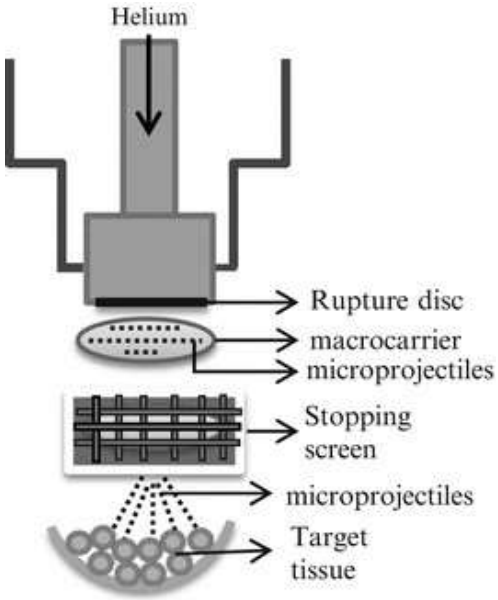
c) BIOLISTICS OR MICROPROJECTILES FOR DNA TRANSFER

Biolistics or particle bombardment is a physical method that uses accelerated micro projectiles to deliver DNA or other molecules into intact tissues and cells. Biolistics transformation is relatively new and novel method amongst the physical methods for artificial transfer of exogenous DNA.

This method avoids the need of protoplast and is better in efficiency. This technique can be used for any plant cells, root section, embryos, seeds and pollen.

Apparatus:

- The gene gun is a device that literally fires DNA into target cells.
- The DNA to be transformed into the cells is coated onto microscopic beads made of either gold or tungsten.
- Beads are carefully coated with DNA. The coated beads are then attached to the end of the plastic bullet and loaded into the firing chamber of the gene gun.
- An explosive force fires the bullet down the barrel of the gun towards the target cells that lie just beyond the end of the barrel.



- When the bullet reaches the end of the barrel it is caught and stopped, but the DNA coated beads continue on toward the target cells.
- Some of the beads pass through the cell wall into the cytoplasm of the target cells. Here the bead and the DNA dissociate and the cells become transformed.
- Once inside the target cells, the DNA is solubilized and may be expressed.

Advantages

- Simple and convenient method involving coating DNA or RNA on to gold microcarrier, loading sample cartridges, pointing the nozzle and firing the device.
- No need to obtain protoplast as the intact cell wall can be penetrated.
- Manipulation of genome of sub-cellular organelles can be done.
- Eliminates the use of potentially harmful viruses or toxic chemical treatment as gene delivery vehicle.
- This device offers to place DNA or RNA exactly where it is needed into any organism

Disadvantages

- The transformation efficiency may be lower than *Agrobacterium- mediated transformation*.
- Specialized equipment is needed. Moreover the device and consumables are costly.
- Associated cell damage can occur.
- The target tissue should have regeneration capacity.
- Random integration is also a concern.
- Chances of multiple copy insertions could cause gene silencing.

d) Lipofection or Liposome GeneDelivery

- Lipofection is a method of transformation using liposomes.
- Liposomes are artificial phospholipid vesicles used for the delivery of a variety of molecules into the cells. They may be multi-lamellar or uni lamellar vesicles with a size range of0.1 to 10 micrometer or 20-25 nanometers respectively.
- They can be preloaded with DNA by two common methods- membrane-membrane fusion and endocytosis thus forming DNA- liposome complex. This complex fuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are susceptible to lipofection method.
- Liposomes can be classified as either cationic liposome or pH-sensitive.

Cationic liposomes are positively charged liposomes which associate with the negatively charged DNA molecules by electrostatic interactions forming a stable complex.

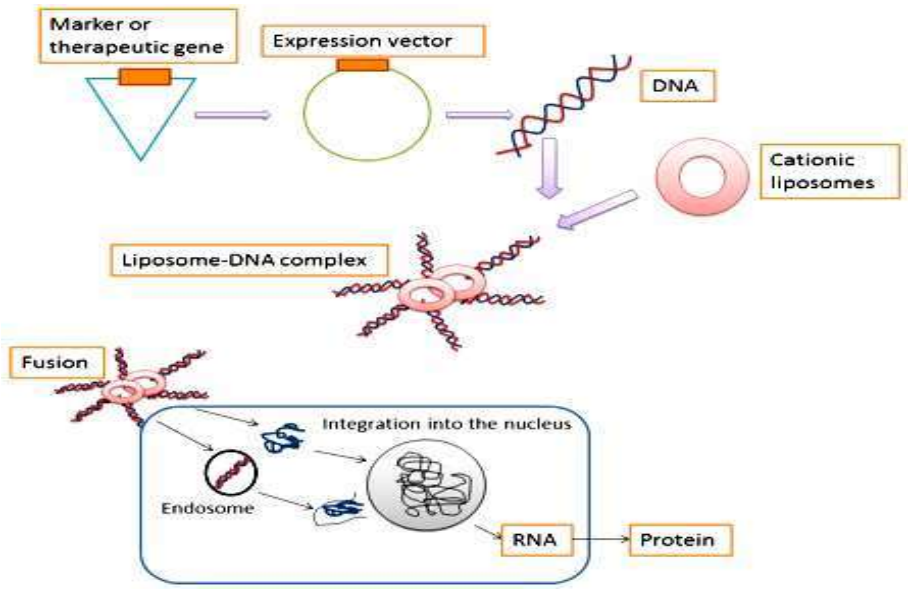
Neutral liposomes are generally used as DNA carriers and helpers of cationic liposomes due to their non-toxic nature and high stability in serum.A positively charged lipid is often mixed with a neutral co-lipid, also called helper lipid to enhance the efficiency of gene transfer by stabilizing the liposome complex (lipoplex). Dioleoylphosphatidyl ethanolamine (DOPE) or dioleoylphosphatidyl choline (DOPC) are some commonly used neutral co-lipids.

The negatively charged DNA molecule interacts with the positively charged groups of the DOPE or DOPC. DOPE is more efficient and useful than DOPC due to the ability of its inverted hexagonal phase to disrupt the membrane integrity.

The overall net positive charge allows the close association of the lipoplex with the negatively charged cell membrane followed by uptake into the cell and then into nucleus.

Negatively charged liposomes

Generally pH-sensitive or negatively-charged liposomes are not efficient for gene transfer. They do not form a complex with it due to repulsive electrostatic interactions between the phosphate backbone of DNA and negatively charged groups of the lipids. Some of the DNA molecules get entrapped within the aqueous interior of these liposomes.



However, formation of lipoplex, a complex between DNA and anionic lipidscan occur by using divalent cations (e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ba^{2+}) which canneutralize the mutual electrostatic repulsion. These anionic lipoplexes comprise anionic lipids, divalent cations, and plasmid DNA which are physiologically safe components.

They are termed as **pH sensitive** due to destabilization at low pH.

The efficiency of both *in vivo* and *in vitro* gene delivery using cationic liposomes is higher thanthat of pH sensitive liposomes. But the cationic liposomes get inactivated and unstable in the presence of serum and exhibit cytotoxicity. Due to reduced toxicity and interference from serum proteins, pH-sensitive liposomes are considered as potential gene delivery vehicles than the cationic liposomes.

Advantages

- Economic
- Efficient delivery of nucleic acids to cells in a culture dish.
- Delivery of the nucleic acids with minimal toxicity.
- Protection of nucleic acids from degradation.
- Measurable changes due to transfected nucleic acids in sequential processes.
- Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

Disadvantages

- It is not applicable to all cell types.
- It fails for the transfection of some cell lines with lipids.

e)Viral mediated genedelivery

Virus mediated gene delivery utilizes the ability of a virus to inject its DNA inside a host cell and takes advantage of the virus' own ability to replicate and implement their own genetic material.

Viral methods of gene delivery are more likely to induce an immune response, but they have high efficiency.Transduction is the process that describes virus-mediated insertion of DNA into the host cell. Viruses are a particularly effective form of gene delivery because the structure of the virus prevents degradation via lysosomes of the DNA it is delivering to the nucleus of the host cell.

In gene therapy a gene that is intended for delivery is packaged into a replication-deficient viral particle to form a viral vector.

Advantages

- High capacity for foreign DNA.
- The vector has no intrinsic cytotoxic effects.

RNA-based viral vectors

RNA-based viruses were developed because of the ability to transcribe directly from infectious RNA transcripts. Gene integration leads to long-term transgene expression but RNA-based delivery is usually transient and not permanent Retroviral vectors include oncoretroviral, lentiviral and human foamy virus.

DNA-based viral vectors

DNA-based viral vectors are usually longer lasting with the possibility of integrating into the genome.DNA-based viral vectors include Adenoviridae, adeno-associated virus and herpes simplex virus.

Herpes virus vectors

• The herpes viruses are linear ds-DNA viruses of approximately 150 kb size *e.g.* EBV (Epstein–Barr virus) and the HSVs (Hepatitis B virus, e.g. HSV-I, varicella zoster).

• Most HSVs are transmitted without symptoms (varicella zoster virus is exceptional) and cause prolonged infections.

• With the help of two viral glycoproteins, gB and gD, the virus binds to cells through an interaction with heparan sulphate moieties on the cell surface.

• Unlike EBV as a replicon vector (contains both *cis* and *trans* acting genetic elements required for replication), HSV-I have been developed as a transduction vector for purpose of gene transferand can efficiently transduce a wide range of cell types.

• HSV virus is remarkably neurotropic and thus HSV vectors are particularly suitable for gene therapy in the nervous system.

HSV can also be transmitted across neuronal synapses during lytic infections which can be used to trace axon pathways.

• Generation of recombinants in transfected cells takes place by homologous recombination. These viral vectors may be replication competent or helper dependent.

• The plasmid based amplicon vectors carrying only the *cis* -acting elements required for replication and packaging can be constructed. These vectors require packaging systems to provide the missing functions in *trans*.

Role in gene therapy

Most promised use of HSV vectors involves gene transfer to neural cells where it can cause a latent infection (e.g. spinal cord, brain, and peripheral nervous system).

Disadvantages

- No viral integration into host genome and transient transgene expression
- High level of pre-existing immunity
- Cytotoxicity effects
- Risk of recombination with latently HSV-infected cells.

DISCUSS ARTIFICIAL INSEMINATION IN DETAIL.

Artifical insemination

In this process, semen collected from a selected male is injected into the uterus or vagina or cervix or fallopian tubes of a female by means of a syringe or a catheter made for that purpose. This facilitates the fusion of gametes where

(i) Male factor infertility

(ii) Unexplained infertility

(iii) Needing to use donor sperm to get pregnant are involved

Two different artificial insemination procedures are in practice:

(a) Intra uterine insemination (IUI) which is most common in use now a days.

(b) Intra cervical insemination (ICI), also known as intra vaginal insemination (IVI) Procedure: For a successful artificial insemination, the following protocol isadapted.

Step-1:The physical examination including blood testing of both wife and husband (or the potential donor) is absolutely necessary.

The physician will do a sperm analysis by obtaining a sperm sample from male partner (or sperm donor). If the partner has ejaculation problems, the doctor can bypass this by extracting sperm from his bladder after ejaculation. The doctor can then use this sperm in the process of artificial insemination.

In case of low sperm counts, he may give sperm samples from ejaculation, if needed.

Step-2:Fertility drugs and monitoring:

The doctor may prescribe fertilitydrugs like Clomid or gonadotropins/injectables (if needed) before the commencement of artificial insemination. Combination of hormonal treatments and IUI seems to give successful results leading to a better chance of pregnancy.

Human chorionic gonadotropin (hCG) may also be administered before the artificial insemination to trigger ovulation. The injection of sperm collected above should then be done within 24-36 hours after the injection of hCG.

Fertility medications will be closely monitored with blood tests and ultrasounds to make sure that the fertilized eggs are developing in a healthy way.

Tracking of ovulatory cycles through the observation of daily basal body temperature and cervical mucus is also important.

Testing of LH surge in urine will indicates ovulation and hence is important for deciding the day and time of artificial inseminations.

Step-3: Preparing the sperm:

In case of IUI, the sperm sample requires a special washing and processing. After a semen sample is obtained, the sperm are washed and concentrated down to maximize the chances of conception.

During this washing and processing phase, potentially toxic chemicals are removed, along with a seminal plasma shell surrounding each sperm cell.

Sperm wash helps in:

(i) sperm concentration to boost the chances of getting pregnancy.

(ii) preventing from possible allergic response to the sperm.

(iii) minimizing any uterine cramping.

Step-4: Insertion of the sperm:

The artificial insemination procedure is quick and usually painless. The female will lie down for a pelvic exam. A speculum will be inserted and the sperm sample will be prepared for insertion. The sperm is placed all the way into the uterine cavity with a thin catheter and syringe. For those using ICI, the sperm will be placed into the vagina and deposited into the cervix with a soft catheter.

Step-5: Rest:

After the artificial insemination procedure, a cervical cap o sponge is placed into the vagina to keep the sperm near the cervix. This sponge or cap can be removed several hours after the AI followed by a rest for 15-20 as it boosts the chances of getting pregnancy.

Many women today use donor sperm during the artificial insemination

procedure for the following reasons:

1. Poor quality of the sperm of the partner.

2. To prevent the Partner to pass on the genetic disorders he is possessing.

3. for a single women or lesbian couples to build a family of their own.

Counselling to reduce anxiety and tension, knowing the information relating to the semen of either the partner or the donor are necessary before the commencement of the protocol.

Success rates for artificial insemination vary basing upon age of the woman, quality of egg or sperm, severity of endometriosis, damage to fallopian tubes due to chronic infection or the blockage of fallopian tubes as IUI will not work in such cases.

Techniques involved during and after artificial Insemination include Cryopreservation of embryos, Embryo Transfer, In Vitro Fertilization, Sex Determination of Sperm or Embryo, Nuclear Transfer or Cloning, Transgenesis and stem Cells.

WRITE AN ESSAY ON IN VITRO FERTILIZATION.

Introduction:

When the union between egg cell and sperm occurs outside the body in a culture vessel, it is known as in vitro fertilization. This involves collection of healthy ova and sperm cells from healthy females and males, respectively. After fusion the resulting zygotes may be cultured in vitro for a period of time to obtain young embryos, which ultimately are implanted in the uterus of healthy females to complete their development.

The implantation of young embryos developed in vitro or obtained from the uterus of different donor females into the womb of selected females is termed as embryo transplantation.

In Vitro Fertilization in humans different steps involved in the technique are

as follows:

(1) Collection of oocytes,

(2) Stimulation of Ovulation

(3) Collection of sperms,

(4) In vitro fertilization of the oocytes, and

(5) Implantation of the Embryo

1. Collection of Oocytes :

Oocytes arecollected from the females who wants to have baby.In this case females have normal functional ovaries, but defective, damage or blocked, fallopain tubes. As a result, the Oocytes released from the ovary a unable to

migrate to the uterus causing infertility (tubal infertility). But when sterility is due to the absence of or nonfunctional ovaries, the Oocytes will be collected from

donor females. Oocyte recovery from donor females done either during their natural or induced ovulation cycles.

The time of natural ovulation is determined by monitoring the rise in the level of Luteinising hormone (LH) either in urine or in blood, and the ova are recovered .This approach yields only one ovum per female/cycle.

(2) Stimulation of Ovulation:

Ovulation may be simulated by the administration of clomiphene and human Menopausal Gonadotropin(hMG). Human chorionic gonadotropin (hCG) also administered to prevent the inhibition of LH surge by hMG. This approach yields several Oocytes from a female, which increases the chances of success of in vitro fertilization.

Follicle development can be arrested at the optimum stage by the administration, of hCG so that the ova are not released. The Oocytes can be collected by laproscopy at a convinient time.

(3) Collection of Sperms:

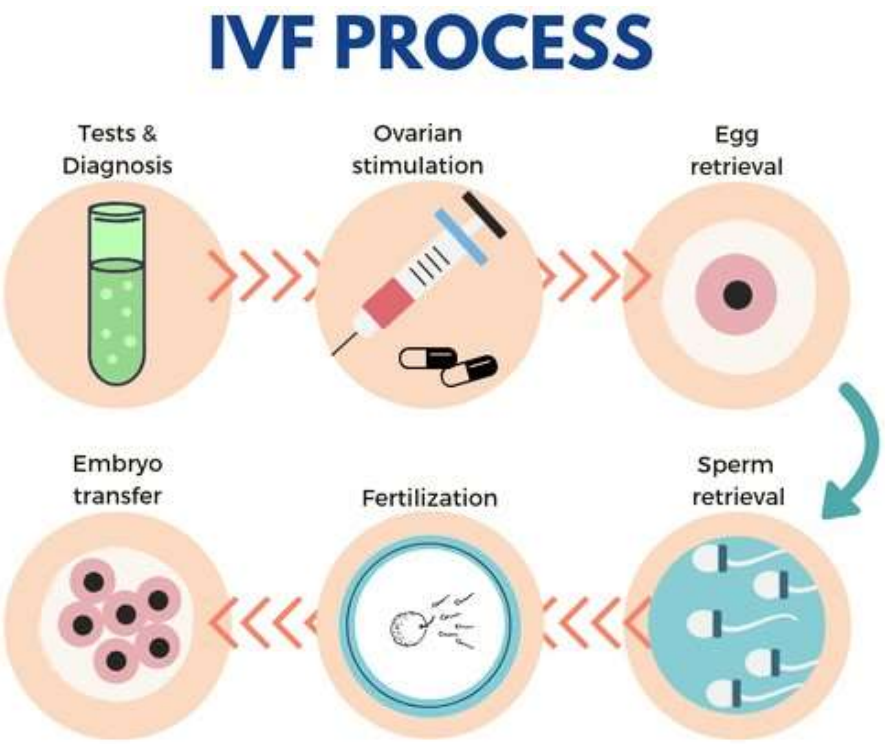
- The semen is collected about 60-90 minutes prior to fertilization.
- It is then liquefied and centrifuged. The sperm pellet so formed is resuspended in culture medium, and incubated for 30-60mm at 37°C.
- A sampled spermatozoa is taken from the surface of the medium for fertilization since the most active sperms are located there.
- The semen is collected from the prospective father.If this is not possible either due to a low sperm count of the semen (oligospermia) due to a lack of motile sperms (azoospermia), semen may be collected from a suitable donor.

(4) In-vitro Fertilizationof Oocytes:

Oocytes are identified by microscopic examination of the follicular fluid aspirated during laproscopy, and are incubated for 5-10hrs depending upon the expected time of their maturation. One of the following media may be used for Oocyte incubation, in vitro fertilization and the subsequent culture of the zygote.

- Modification of Ham's F-10 medium,
- Earl's solution
- Modified whitten's medium, and

- Whittingham's T medium.



Fertilization is effected by adding 10,000-50,000 motile sperms to about 100% to 1 ml of culture medium in which the Oocyte is being incubated. The Oocyte examined after 12-13 hours for the detection of following.

(1) Number of pronuclei, and polar bodies,

(2) Granulation of the Oocyte and

(3) The shape of Oocyte.

(5) Implantation of the Embryo

7-8 days after fertilization, the embryo is implanted into the female reproductive tract. This woman acted like a surrogate mother.

A normally fertilized oocyte contains two pronuclei and two polar bodies. Any departure from this and the presence of granulation and abnormalities in shape are indicative of abnormalities in the zygotes, and such zygotes are rejected.

The first division in the zygote occurs about 24-30 hours after insemination, but each subsequent division takes about 10-12 hours. Therefore, if an oocyte fails to divide by about 30 hours after insemination, it should not be used for implantation.

Advantages of IVF:

- IVF helps many patients who would be otherwise unable to conceive.
- *It is helpful for female* Older patients with a low ovarian reserve*,Endometriosis,Premature ovarian failure.*
- Males with infertility *conditions like oligospermia and azoospermia* Unexplained infertility.
- IVF can help to diagnose fertilization problems.

Disadvantages

- An IVF cycle may be unsuccessful.
- There may be associated side effects and risks.
- Multiple pregnancy.
- IVF treatment can be expensive.

EXPLAIN THE PRODUCTION OF TRANSGENIC FISH. ADD A NOTE ON IT'S IMPORTANCE.

Introduction:

Transgenesis refers to the process of introducing transgene (i.e. an exogenous gene) from one organism into another. So that organism will exhibit new property and transmit that property to its off-spring.

A transgenic fish is an improved variety of fish provided with one or more desirable foreign gene for the purpose of enhancing fish quality, growth, resistance and productivity.

Medaka fish (Japanese Rice Fish) has several characteristics, well suited for the studies in environmental toxicology, histopathology, carcinogenesis, germ cell mutagenesis etc.

Technique :

Attempts to produce transgenic fish started in 1985 and some encouraging results have been obtained. The genes that have been introduced by microinjection in fish include the following.

(1) Human or Rat gene for growth hormone

(2) Chicken gene for delta - crystalline protein.

(3) E. Coli gene for β-galactosidase.

(4) E. Coli gene for neomycin resistance.

(5) Winter flounder gene for antifreeze protein.

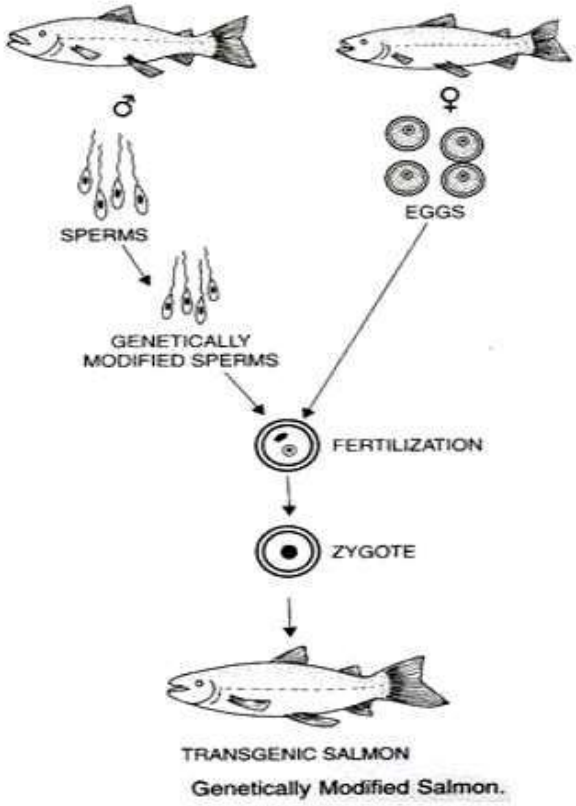
(6) Rainbow trout gene for growth hormone.

The technique of microinjection has been successfully used to generate transgenic fish in many species such as common carp, cat fish, goldfish, loach medaka, salmon, tilapia, rainbow trout and zebra fish.

In most fish species studied so far, pronulcei could not be easily visualized. Hence the DNA needs to be injected into the cytoplasm.

- Eggs and sperms from mature individuals are collected and placed into a separate dry container.
- Fertilization is initiated by adding water and sperm to eggs, with gentle stirring to facilitate the process of fertilization.
- Egg shells are hardened in water. About 10% to 10 molecules of linearized DNA in a volume of 20ml or less are microinjected into each egg.
- Within the first few hours after fertilization. Following micro injection, eggs are incubated in appropriate hatching trays and dead embryos are removed daily.
- Human growth hormone gene transferred to transgenic fish allowed growth that was twice the size of their corresponding non - transgenic fish.

- Similarly Anti Freeze Protein (APP) gene was transferred in several cases and its expression was studied in transgenic salmon. It was shown that the level of APP gene expression is still too low to provide protection against freeze.
- In the production of transgenic zebra fish, a plasmid containing rat-growth hormone gene was microinjected into fertilized eggs of the Zebra fish and its presence was confirmed in adult fish.



Genetically Modified Salmon.

Transgenic fish may be better used one in the following purposes:

(1) For increasing fish production to meet the growing demand for food from increasing world population.
(2) For production of pharmaceutical and other industrial products of piscine origin.
(3) For development of transgenic native glow fish varieties for aquarium.

(4) As fish biosensors for monitoring aquatic pollution.
(5) For isolation of genes, promoters and synthesis of effective gene constructs.
(6) For researches in embryonic stem cells and in-vitro embryo production.
(7) For production of anti-freeze protein.

Transgenic fish developed:

The first transgenic fish were produced in 1995. About 35 species had been genetically engineered subsequently worldwide. Among the major food fish species are carp, tilapia, salmon, medaka and goldfish are used in basic research.

Strategies adapted to produce Transgenic fish:

Methods applied for the productions of transgenic fish are Microinjection, Electroporation, Sperm mediated gene transfer, Retroviral infection.

Advantages of transgenic fish:

- Transgenic fish is used for the production of human therapeutics, experimental models for biological research, environmental monitoring, ornamental fish and aqua cultural production.
- Transgenic strains provide useful model systems for studying the consequences of growth enhancement from genetic, physiological and ecological standpoints.
- Commercial production of transgenic fish able to transmit desirable characteristics, such as enhanced growth or disease resistance to their progeny.
- Zebra fish, Atlantic salmon showed faster growth in each generation with
  greater efficiency of feed conversion.
- Use of transgenic fish as bioreactors for the large-scale production of rare human therapeutic proteins or novel foods for specific dietary requirements is in practice.
- Transgenic lines of tilapia engineered to produce human clotting factor VII is used in liver transplants and in treating injuries.

Disadvantages of transgenic fish:

- Loss of genetic diversity, loss of biodiversity, and reduction in species richness are the identified disadvantages.
- Transgenic fish are those related to their involuntary escape into environment.
- Extremely fast-growing transgenic salmon and loach have low fitness and die inefficient process.
- Transgenic fish could produce new or modified proteins that could be toxic to humans.
- The transgenic fish are also more active and aggressive when feeding, and more willing to risk exposure to predation.
- The transgenic salmon pose serious ecological threats to wild populations and are not engineered for natural environments.
- Transgenesis can result in an unpredictable number of copies and site of fo integration of the early.

SHORT ANSWER QUESTIONS

$P^{BR}322$

The **pBR322** was known to be the first artificial cloning vector to be constructed.It is a plasmid which is a part of an extra chromosomal DNA segment, it helps in the cloning of the vector and also is important in genetic engineering.It is second generation plasmid vector.

The **p** stands for "plasmid," and **BR** for "Bolivar" and "Rodriguez, who constructed this plasmid.

322 - It is a number that was assigned to distinguish this plasmid from others developed in the same laboratory.

It contains CoIE1 origin of replication control. Generally there are 15-20 molecules present in a transformed E.Coli cell,but this number can be amplified by incubating a log phase culture of CoIE1 carrying cells in the presence of Chloramphenicol.

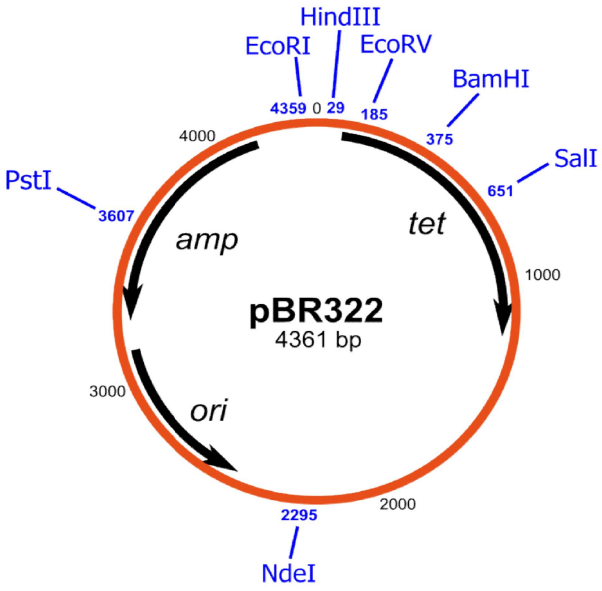
pBR322 is found to have around 4361 base pairs in length with the two antibiotic resistance genes that are associated with it. The two antibiotic genes are named as,

The gene **bla** that helps in the encoding of the the Ampicillin resistance (*AmpR*) protein,

The gene **tetR** that helps in the encoding of the Tetracycline resistance (*TetR*) protein.

The replicon rep responsible for the replication of plasmid.
rop gene coding for the Rop protein, which promotes conversion of the unstable RNA I - RNA II complex.

This plasmid also constitutes the region called the origin of replication of pMB1 where the cloning starts and the rop gene that helps in the encoding of the restrictor prevents the extra plasmid copy number.



The complete nucleotide sequence of $P^{BR}322$ has been determined. The plasmid contains 20 unique recognition sites for restriction enzymes,

Six of these sites are ECoR V, BamHI, SphI, SalI, XmaIII and Nrul are located with in the genes coding for the Tetracycline resistance.

Two sites in this vector for the restriction enzymes HindIII and Cla I located within the promoter of the TetR gene.

Three sites for **PstI**, **PvuI** and **ScaI** are lie with in the Lactamase gene.

Cloning of DNA fragment into any of these 11 sites results in the insertional inactivation of either one of the antibiotics resistance markers.The gene of interest is spliced into tet R gene cluster, and then the E.Coli cells are transformed.

TRANSGENIC SHEEP

Experiments done by J.P. Simons of Edinburg concluded that, for the first time,Two transgenic ewes were produced, each carrying about 10 copies of human anti-hemophilic factor IX gene fused with the BLG gene (BLG = β-lactoglobulin).

In another report, also from Edinburgh, five transgenic sheep were produced. In all these cases, transgene involved fusion of the ovine β-lactoglobulin gene promoter fused to the human α_1 antitrypsin (hα,AT) gene. Four of these animals were female and one was male.

Method of transgenesis:

- Sheep fibroblasts growing in tissue culture were treated with a vector.
- 2 regions of DNA homologous to the sheep **COL1A1 gene** (Collagen,type I, alpha 1) encoding for Type 1 collagen.This locus was chosen because fibroblasts secrete large amounts of collagen.
- A neomycin-resistance gene is added to aid in isolating those cells that successfully incorporated the vector. The human gene encoding alpha1-antitrypsin causes damage to the lungs and liver.
- Promoter sites were identified from the beta-lactoglobulin gene expressing itself in milk-producing cells. Binding sites help the ribosomes for efficient translation of the beta-lactoglobulin mRNAs.
- Successfully-transformed cells were then fused with enucleated sheep eggs and implanted in the uterus of a ewe (female sheep). Several embryos survived until their birth, and two young lambs lived over a year. When treated with hormones, these two lambs secreted milk containing 650 ug/ml of alpha l-antitrypsin (50 times higher than earlier results).

- Recombinant DNA technique can also be used to increase the ability of sheep for wool growth. For this purpose, genes essential for synthesis of some imp amino acids found in keratin proteins of wool, have been cloned and introduced in embryos to produce transgenic sheep.
- For instantce, genes for two enzymes (serine acetyltransferse SAT and O-acetylserine sulfhydrylase = OAS), involved in cysteine biosynthesis, were isolated from bacteria and cloned in a vector. These genes were introduced in sheep cells, ultimately leading to the production of transgenic sheep expressing these genes.
- Genes for growth hormone have also been introduced and can be used to promote body weight other genes involved in wool production have also been cloned and will be used for transgenesis, thus increasing the potential of wool production through genetic engineering.

Applications of transgenic Sheep :

1. It is used as a model for studying.
2. Immunology.
3. Human blood clotting factor viii.
4. Transplantation.
5. Haematology.
6. Biological product manufacturing.
7. Recombinant DNA.
8. Drug production in milk.

SUPER OVULATION

Superovulation is defined as the drug-induced production of multiple eggs for use during assisted reproductive technologies such as in vitro fertilization (IVF). Superovulation is often used in combination with other fertility treatments, to increase a woman's chances of becoming pregnant successfully. Superovulation is also called controlled ovarian stimulation.

Normally, a woman ovulates just one egg per cycle. With the use of fertility drugs, she may be able to produce several eggs, which can then be retrieved from the ovaries prior to ovulation. **Clomid** is the medicine commonly used to induce ovulation in females.

Risks of superovulation include Ovarian Hyperstimulation Syndrome (OHS), Ovarian Torsion, and Multiple (twins, triples, etc.) Pregnancy.

Women with open fallopian tubes and whose partners have adequate sperm counts are candidates for superovulation.

- If a woman already ovulates and is not conceiving,
- If a woman has been ovulating with an oral medication (such as clomiphene) and is not conceiving, superovulation can improve her chances of conceiving by stimulating her ovaries to release more eggs.

Procedure:

- Generally an ultrasound at the time of ovulation is done to know the growing number of ovarian follicles are growing.
- If the ultrasound reveals that the woman is producing just one follicle, the dose or medication might be changed in the next cycle.
- Many women undergoing superovulation will opt for gonadotropins.
- Gonadotropins are hormones causing eggs to grow. These medicines are given by injection with a small needle, just beneath the skin.
- Gonadotropins like,Bravelle, Follistim, Gonal-F, Menopure and Repronex, are available,but most are equally effective. A woman using gonadotropins requires close monitoring to ensure that she is not producing too many eggs.
- Once the desired number of eggs mature, the woman is given a human chorionic gonadotropin (hCG) injection to cause ovulation. If she is doing intrauterine insemination (IUI), she will return to the clinic 36 hours after the hCG injection for the insemination.

Side effects:

- Multiple births, Ovarian hyperstimulation syndrome (OHSS),bloating and pelvic discomfort are the common side effects observed during superovulation.
- Difficulty in urination, weight gain, dehydration involving complications in the lungs, kidneys and liver.

- Low body weight, Age, Polycystic ovary syndrome (PCOS) result because of higher dosage of gonadotropins. Rarely ectopic pregnancy and ovarian cancer are diagnosed.

UNIT – V

LONG ANSWER QUESTIONS

WRITE AN ESSAY ON PCR TECHNOLOGY AND IT'S APPLICATIONS

PCR or Polymerase Chain Reaction is a technique used in molecular biology to create several copies of a certain DNA segment. This technique was developed in 1983 by Kary Mullis, an American biochemist. PCR has made it possible to generate millions of copies of a small segment of DNA. PCR is used in many areas of biology and medicine, including molecular biology research, medical diagnostics, and even some branches of ecology.

Principle of PCR:

The PCR technique is based on the enzymatic replication of DNA. In PCR, a short segment of DNA is amplified using primer mediated enzymes. DNA Polymerase synthesis new strands of DNA complementary to the template DNA. The DNA polymerase can add a nucleotide to the pre-existing 3'-OH group only. Therefore, a primer is required. Thus, more nucleotides are added to the 3' prime end of the DNA polymerase.

Components of PCR:

Components of PCR constitutes the following

1. **DNA Template**– The DNA of interest from the sample.

2. **DNA Polymerase**– Taq Polymerase is used. It is thermostable and does not denature at very high temperatures.

3. **Oligonucleotide Primer**s- These are the short stretches of single-stranded DNA complementary to the 3' ends of sense and anti-sense strands.

4.**Deoxyribonucleotide triphosphate**– These provide energy for polymerization and are the building blocks for the synthesis of DNA. These are single units of bases.

5. **Buffer System**– Magnesium and Potassium provide optimum conditions for DNA denaturation and renaturation. It is also important for fidelity, polymerase activity, and stability.

6. Divalent cations: They act as a co-factor for Taq polymerase which increases its polymerase activity. Generally Mg^{2+} is used, but Mn^{2+} can be applied to achieve PCR-mediated DNA mutagenesis. This is because higher Mn^{2+} concentration leads to higher error rate during DNA synthesis.

Procedure:

Typically, PCR is designed of 20-40 repeated thermal cycles,with each cycle consisting of 3 discrete temperature steps:

1.Denaturation,

2.Annealing and

3.Elongation

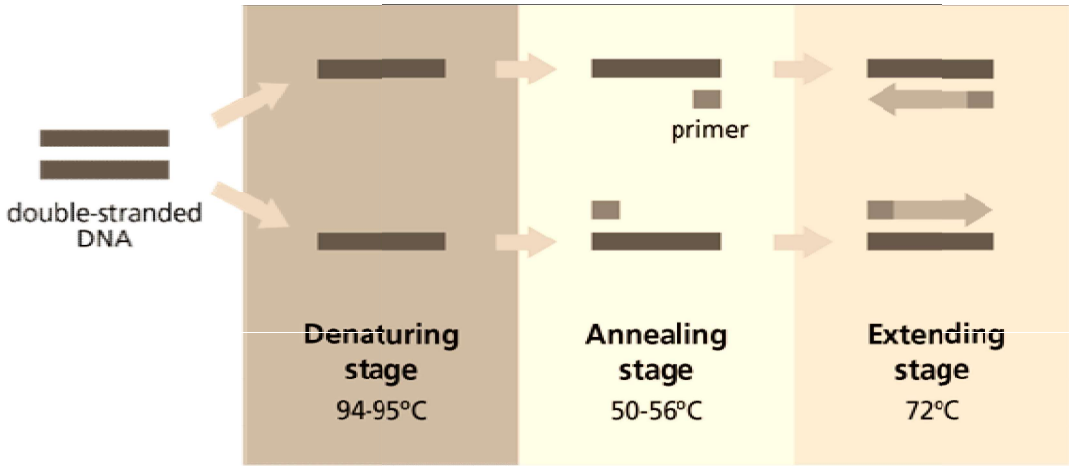
1.Denaturation

Denaturation occurs when the reaction mixture is heated to 94℃ for about 0.5 to 2 minutes. This breaks the hydrogen bonds between the two strands of DNA and converts it into a single-stranded DNA.

The single strands now act as a template for the production of new strands of DNA. The temperature should be provided for a longer time to ensure the separation of the two strands.

a) Initial Denaturation

b) Denaturation



a) Initial denaturation:

Initial denaturation involves heating of the reaction to a temperature of 94–96°C for 7-10 minutes (or 98°C if extremely thermostable polymerases are used).For specifically engineered DNA polymerases (Hot start Taq polymerases) activity requires higher range of temperature.

The initial heating for such a long duration also helps in gradual and proper unfolding of the genomic DNA and subsequent denaturation, and then exposing target DNA sequence to the corresponding primers.

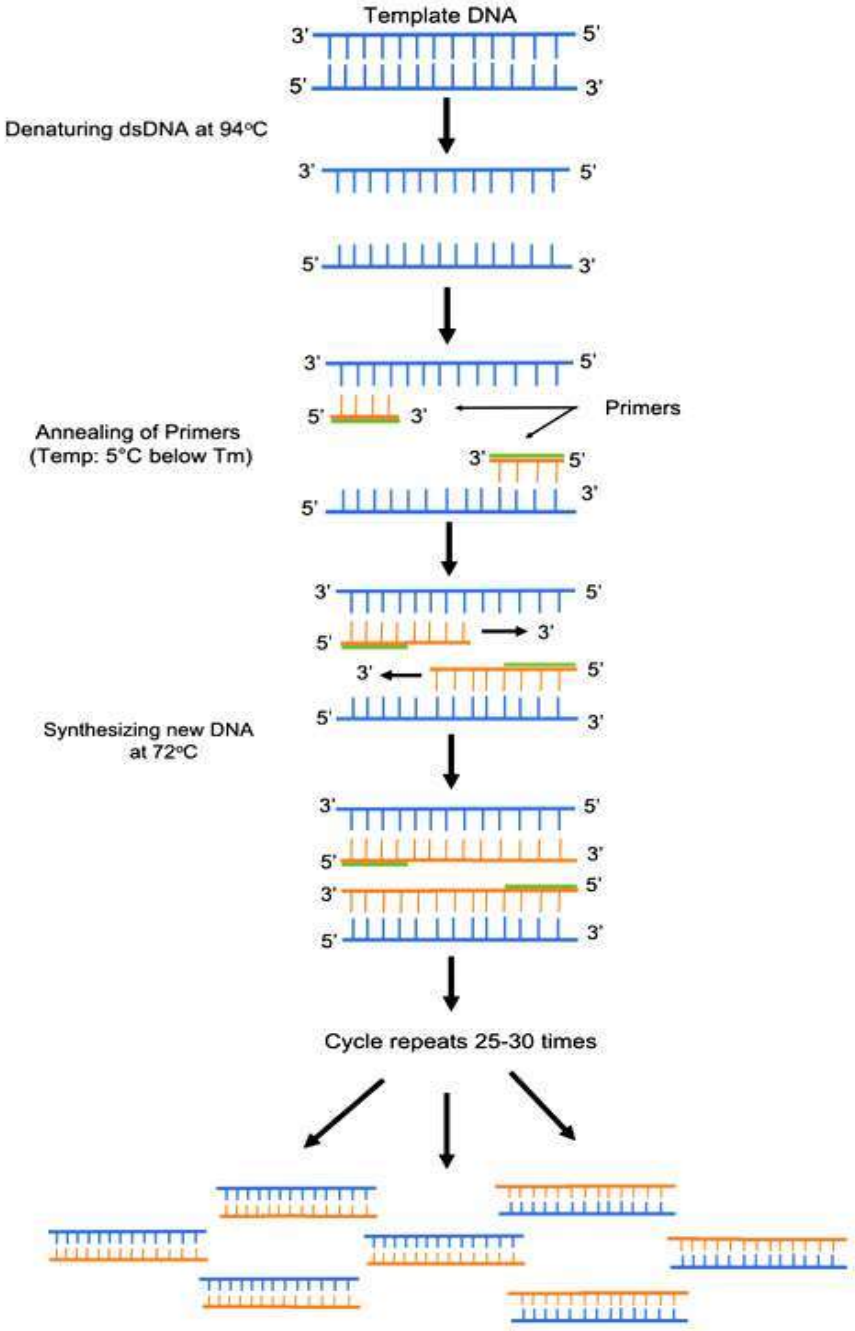
b) Denaturation:

Denaturation requires heating the reaction mixture to 94–98°C for 20–30 seconds. It results in melting of the DNA template by breaking the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

2.Annealing

After the separation of the two strands of DNA,the temperature of the reaction mix is lowered to 50–65°C for 20–50 seconds to allow annealing of the primers to the single-stranded DNA templates. Typically the annealing temperature should be about 3-5°C below the T_m of the primers.

Stable complimentary binding are only formed between the primer sequence and the template when there is a high sequence complimentarity between them.


Template DNA
3'
5'
5'
3'
Denaturing dsDNA at 94°C
3'
5'
5'
3'
3'
5'
5'
3'
Primers
Annealing of Primers
(Temp: 5°C below Tm)
3'
5'
5'
3'
3'
5'
5'
3'
3'
5'
5'
3'
Synthesizing new DNA
at 72°C
3'
5'
5'
3'
3'
5'
5'
3'
Cycle repeats 25-30 times

3.Elongation

At this step, the temperature is raised to 72-80℃. The bases are added to the 3' end of the primer by the Taq polymerase enzyme.This elongates the DNA in the 5' to 3' direction. The DNA polymerase adds about 1000bp/minute under optimum conditions.Taq Polymerase can tolerate very high temperatures. It attaches to the primer and adds DNA bases to the single strand. As a result, a double-stranded DNA molecule is obtained.These three steps are repeated 20-40 times in order to obtain a number of sequences of DNA of interest in a very short time period.

Applications of PCR:

The following are the applications of PCR :

Medicine

• Testing of genetic disease mutations.

• Monitoring the gene in gene therapy.

•Detecting disease-causing genes in the parents.

Forensic Science

• Used as a tool in genetic fingerprinting.

• Identifying the criminal from millions of people.

• Paternity tests.

Research and Genetics

• Compare the genome of two organisms in genomic studies.

• In the phylogenetic analysis of DNA from any source such as fossils.

• Analysis of gene expression.

• Gene Mapping.

Others

The Human Genome Project (HGP) for determining the sequence of the 3 billion base pairs in the human genome, relied heavily on PCR.

PCR can help to study for DNA from various organisms such as viruses or bacteria. PCR has been used to identify and to explore relationships among species in the field of evolutionary biology.

In Anthropology, it is also used to understand the ancient human migration patterns.
In Archaeology, it has been used to spot the ancient human race.
PCR commonly used by Paleontologists to amplify DNA from extinct species or cryopreserved fossils of millions years.

DESCRIBE AUTOMATED DNA SEQUENCING METHOD.

"A laboratory technique employed to known the correct DNA sequence by the sequential chemical reaction is known as DNA sequencing."

Sanger sequencing also known as the "chain termination method," was developed by the Frederick Sanger and his colleagues in 1977. This method is designed for determining the sequence of nucleotide bases in a piece of DNA (commonly less than 1,000 bp in length). Sanger sequencing with 99.99% base accuracy is considered the "gold standard" for validating DNA sequences, including those already sequenced through next-generation sequencing (NGS).

Sanger Sequencing Steps

The Sanger sequencing method consists of 6 steps:
(1) The double-stranded DNA (dsDNA) is denatured into two single-stranded DNA (ssDNA).
(2) A primer that corresponds to one end of the sequence is attached.
(3) Four polymerase solutions with four types of dNTPs but only one type of ddNTP are added.
(4) The DNA synthesis reaction initiates and the chain extends until a termination nucleotide is randomly incorporated.
(5) The resulting DNA fragments are denatured into ssDNA.
(6) The denatured fragments are separated by gel electrophoresis and the sequence is determined.

Automated DNA Sequencing

The manual Sanger method was tedious.The semi-automated Sanger sequencing method is based on the principle of Sanger's method with some minor variations.

Instead of the 4 different reactions, the automated DNA sequencing is carried out in a single tube. This means on a gel the DNA runs in a single lane.

Here in the semi-automated DNA sequencing, the fluorescent-labeled set of primers are used, instead of ddNTPs. Thus four different primers give four different peaks.

The PAGE method isn't capable of separating all the fragments in a single reaction. Therefore, alternatively, the capillary gel electrophoresis method is practiced. This method separates each and every single fragment precisely

By using the labeled primers or the dNTPs, the machine reads the sequence accurately, on a single lane capillary electrophoresis. We can sequence more than 300 samples in a single run on an automated DNA sequencing platform.

The capillary electrophoresis is used to separate DNA molecules on the basis of size, It is powerful enough to separate a single base pair fragment. The chromatogram generated through the C.E sent the output as a fluorescent peak.

Pyrosequencing

In 1993, *Bertil Pettersson, Mathias Uhlen* and *Pål Nyren* described the pyrosequencing method.

The method is based on the detection of the pyrophosphate released during the chain reaction of nucleotide addition. Here the order of the nucleotide is determined by the PPi released during the joining of two adjacent nucleotides (3'OH- 5'P).

In contrast with other methods, instead of a single polymerase, two additional enzymes are required in the pyrosequencing method. The three enzymes are:

- DNA polymerase (without exonuclease activity)
- Luciferase
- Sulfurylase

All three enzymes work in a sequential manner for the detection of the PPi. The real-time polymerase activity monitoring allows the detection of the released pyrophosphate in a cascade of the enzymatic reaction,

(DNA)n + dNTP ——————— (DNA)n+1 + PPi **(Polymerase)**

The addition of one dNTP removes one pyrophosphate from the DNA.

PPi + APS ——————— ATP + SO_4^{-2} **(ATP sulfurylase)**

ATP + luciferin + O2 ——————— AMP + PPi + oxyluciferin + CO2 + photon **(luciferase)**

Here the reaction is completed into the three steps:

The enzyme polymerase adds the dNTPs to the single-stranded DNA. If the correct complementary base is added, the pyrophosphate is released.

The enzyme sulfurylase converts the PPi into ATP (energy) with the help of the APS (adenosine 5´ phosphosulfate).

The ATP act as a substrate for the luciferase activity (more specifically “firefly luciferase”). With the help of the ATP substrate, the luciferase converts the luciferin into oxyluciferin in the presence of oxygen and the photon of light is released.

Once the correct nucleotide is added, the amount of the light released by the enzymatic reaction is detected by the charged device coupled camera, photodiode, or a photomultiplier tube. This is the basic fundamental of the pyrosequencing setup.

Based on the substrate used in the technique two types of pyrosequencing methods are available:

Solid-phase pyrosequencing and Liquid phase pyrosequencing.

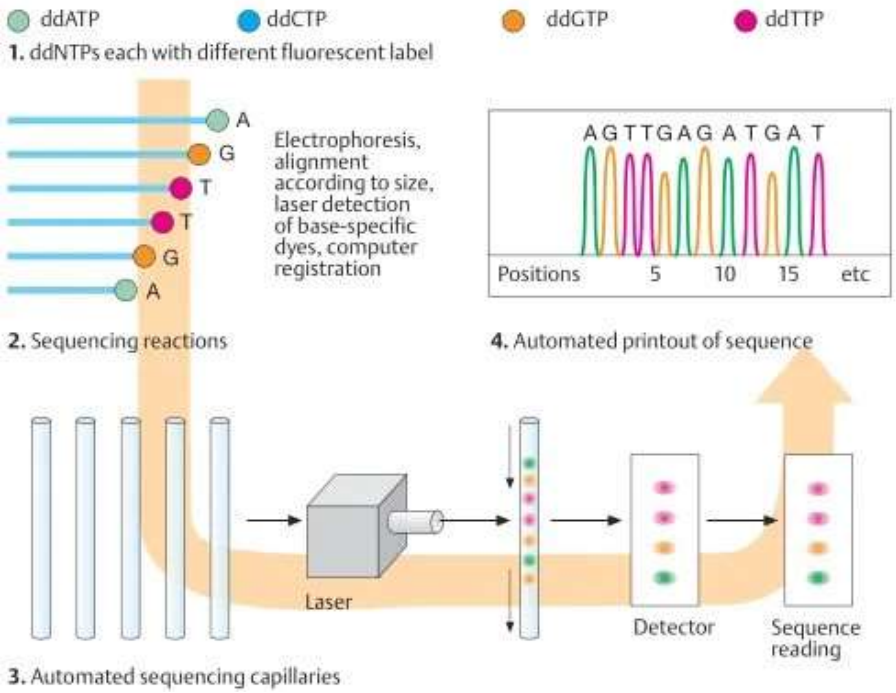
Solid-phase Pyrosequencing

The solid-phase Pyrosequencing is being applied in microfluidic format,which is the first demonstration of sequencing an entire genome.
The microfluidic instrument sequences DNA within thousands of 75 pico- liter wells contained within the specifically designed PicoTiter Plate.The high density PicoTiter plate allows amplification and sequencing of several thousand to several hundred thousand DNA samples simultaneously.

DNA fragments are first amplified within the wells, eliminating the need for offline amplification, which is less cost and time- effective.

Liquid phase Pyrosequencing

Pyrosequencing by the liquid-phase approach came about by the introduction of a nucleotide-degrading enzyme, called apyrase. The implementation of this enzyme in the Pyrosequencing system excluded the use of solid-phase separation, and consequently, eliminated extra steps such as washes and repetitive enzyme additions.



Whole-genome shotgun sequencing

Yet another modification of Sanger's chain termination method is the whole-genome shotgun sequencing. However, instead of a single gene or few base pairs, the present method is powerful enough to sequence the entire genome of an organism.

The principle of the shotgun is the same as Sanger's method, one additional step of DNA fragmentation allows to read multiple fragments.

The entire genome of an organism is fragmented with the help of endonuclease enzymes or by mechanical techniques. After that, the smaller fragments of DNA are sequenced individually into the machine.

The computer-based software analyses each and every overlapping fragment and reassembled it to generate the complete sequence of the entire genome.

The method can be divided into four steps:

1. Fragmentation of a DNA: with the help of restriction endonucleases or physical method
2. Formation of libraries of the subfragments: the fragments are ligated in vectors and an entire library for various vectors are generated
3. Sequencing the subfragments: each library is sequenced individually.
4. Generation and reading the contigs: the overlapping fragments called contigs are read by the computer.

The technique solely depends on computational analysis. A huge, powerful, supercomputer is required to work efficiently.

WRITE AN ESSAY ON SOUTHERN BLOTTING TECHNIQUE AND IT'S USES.

INTRODUCTION

Southern blotting is a method used to transfer DNA from an agarose gel to a membrane, where the DNA can be subsequently probed for a specific sequence. The technique was named after its inventor, Edward M. Southern,as a result subsequent blotting techniques have used similar nomenclature, for example Northern blotting, the transfer of RNA; Western blotting, the transfer of proteins.

PRINCIPLE

▪ Southern blotting is an example of RFLP (restriction fragment length polymorphism). Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.

▪ Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon

membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.

▪ A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

Steps

- Restriction digest: by RE enzyme and amplification by PCR.
- Gel electrophoresis: SDS gel electrophoresis.
- Denaturation: Treating with HCl and NaOH.
- Blotting.
- Baking and Blocking with casein in BSA.
- Hybridization using labelled probes.
- Visualization by autoradiogram.

Step I: Restriction digest

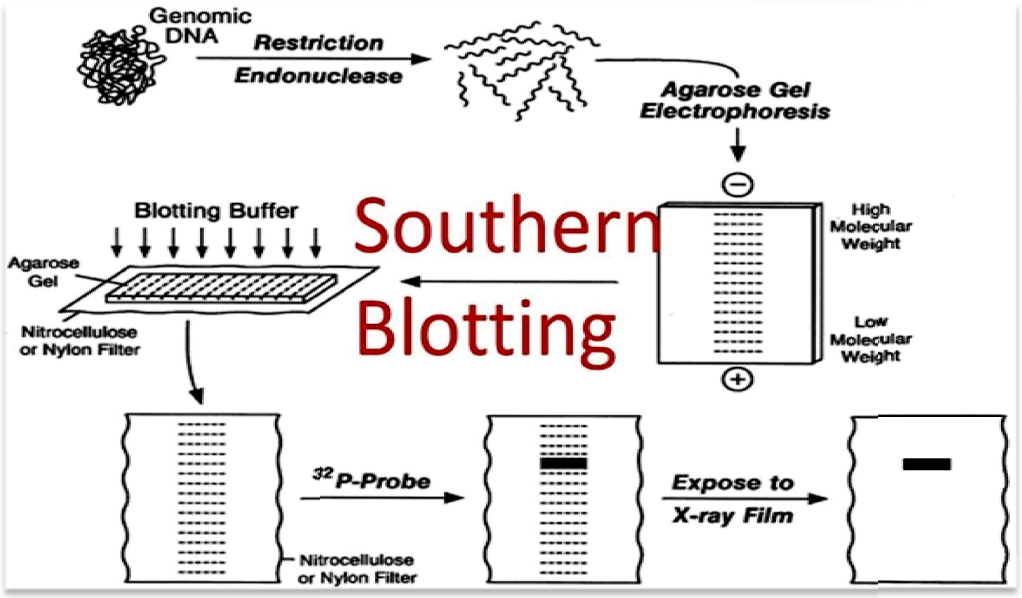
- The DNA is fragmentized by using suitable restriction enzyme. RE cuts the DNA at specific site generating fragments.
- The number of fragments of DNA obtained by restriction digest is amplified by PCR.

Step II: Gel electrophoresis

- The desired DNA fragments is separated by gel electrophoresis.

Step III: Denaturation

- The SDS gel after electrophoresis is then soaked in alkali (NaOH) or acid (HCI) to denature the double stranded DNA fragments.
- DNA strands get separated.



Step IV: Blotting

- The separated strands of DNA are then transferred to positively charged membrane nylon membrane (Nitrocellulose paper) by the process of blotting.

Step V: Baking and blocking

- After the DNA of interest bound on the membrane, it is baked on autoclave to fix in the membrane.
- The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates all the binding site of membrane

Step VI: Hybridization with labelled probes

- The DNA bound to membrane is then treated with labelled probe
- The labelled probe contains the complementary sequences to the gene of interest.

- The probe bind with complementary DNA on the membrane since all other nonspecific binding site on the membrane has been blocked by BSA or casein.

Step VII: Visualization by Autoradiogram

- The membrane bound DNA labelled with probe can be visualized under autoradiogram which give pattern of bands.

Application of Southern blotting:

1. Southern blotting technique is used to detect DNA in given sample.
2. DNA finger printing is an example of southern blotting.
3. Used for paternity testing, criminal identification, victim identification.
4. To isolate and identify desire gene of interest.
5. Used in restriction fragment length polymorphism.
6. To identify mutation or gene rearrangement in the sequence of DNA.
7. Used in diagnosis of disease caused by genetic defects.

DEFINE FERMENTATION.EXPLAIN ABOUT ANY TWO TYPES OF FERMENTATION.

Introduction :

Fermentation is the metabolic process' in which raw materials such as sugar or carbohydrates are converted into economically important products like acids, gases and alcohols by micro-organism. This process is carried out in a equipment called as fermentor.

The microorganisms are grown under suitable conditions, by providing raw materials meeting all the necessary requirements such as carbon, nitrogen, salts, trace elements and vitamins.

The major products of fermentation possess high commercial value are wine, beer, cider, vinegar, ethanol, cheese, hormones, antibiotics, complete proteins, enzymes and other useful food products like bread and cheese.

Basic Steps of Industrial Fermentation :

Industrial fermentation operation can be broken down into three main stages, viz,

Upstream processing,

Fermentation process and

Downstream processing.

Upstream processing:

Upstream processing **comprises tasks in the initial stages of the fermentation process in biotechnology**. This includes all steps related to the development of microorganisms, nutrient preparation, cell culture, cell separation and harvesting.

Fermentation process:

The fermentation process involves the propagation of the micro organism and production of the desired product.

It can be either aerobic fermentation, (presence of oxygen) or anaerobic fermentation, (absence of oxygen). Many industrial fermentation are carried out under aerobic conditions. A few processes such as ethanol production by yeast require strictly anaerobic environments.

The fermentation process can also be divided into three basic systems, depending on the feeding strategy of the culture and the medium in the fermenter namely –

(i) Batch Fermentation: The medium and the culture are initially fed into the vessel and it is then closed. After thatadd acid or alkali for the pH adjustment,in the presence of oxygen.The fermentation is allowed to run for a predetermined period of time. The end product is harvested at the end.

(ii) Continuous Fermentation: In a continuous process, fresh medium is continuously added and the products, along with the culture are removed at the same rate, thus maintaining constant concentrations of nutrients and cells throughout the process.

(iii) Fed-batch Fermentation :A fed-batch system is a combination of these two systems where additional nutrients are added to the fermentor as the fermentation is in progress.

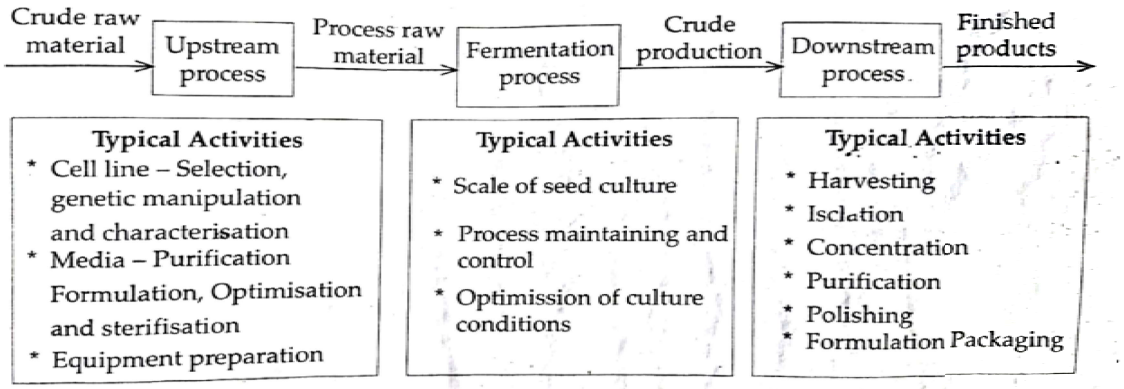
Types of fermentation:

This involves four types viz.,

Alcoholicfermentation,

Lactic acid Fermentation,

Propionic acid fermentation,



Flow chart showing the process of Fermentation

Butyric acid (Butanol fermentation),and

Mixed acid fermentation.

1. Alcoholic Fermentation

- Here the end product is the ethanol (CH_2CH_2OH). Yeast used in the fermentation process is Saccharomyces cerevisiae.
- The energy-yield under anaerobic conditions is much lower resulting in slow growth and less cell-yield.
- In aerobic environment, cell yield increases, but alcohol production falls. It implies that oxygen inhibits fermentation which is termed as **Pasteur-effect.**
- Conversion of pyruvic acid to ethanol occurs in two steps:

(a) Pyruvic acid to acetaldehyde is catalysed by pyruvic acid decarboxylase and this requires TPP as coenzyme.

(b) Formation of ethanol from acetaldehyde by alcohol dehydrogenase requiring$NADH_2$as coenzyme.

2.Lactic acid fermentation: This is of two types viz.,

(i) Homofermentative type in which one molecule of lactic acid is produced by the reduction of one molecule of pyruvic acid. Hence two molecules of lactic acidis formed from one molecule of glucose. The reaction is catalysed by an enzyme Lactic acid dehydrogenase.

(ii) Heterofermentitive type in which from one molecule of glucose, lactic acid and ethanol or acetic acid and CO_2 are formed. The reaction is promoted by lactic acid bacteria viz., like Lactobacillus brevis, Bifidobactrium bifidum Sporolactobacillus etc., prefer anaerobic conditions and yield various fermented food products like curd from milk, cucumber pickles, beef and pork sausages etc.

3. Propionic acid(CH_3CH_2COOH) fermentation:

- Several anaerobic bacteria like Clostridium,Propionibacterium acidipropionici and P. Freudenreichii living in the rumen of cattle possess cytochromes and catalses.
- They act as fermentors and can tolerate some amount of oxygen.

- These Bacteria are used in the commercial production of swiss cheese since a special flavour is added by their interaction.

4. Butyric acid - butanol fermentation:

- Obligative anaerobic spore forming bacteria like Clostridium butyricum, C. lactoacetophilum, C. pasteurianum are the living fermentors directly involved in the production of butanol, isopropanol or acetone besides CO_2.
- Here two molecules of acetyl CoA are condensed by the action of enzymes thiolase to yield acetoacyl CoA.
- This is dehydrogenated by Hydroxybutyryl CoA. Removal of butiryl CoA and addition of water converts the above product to butyl alcohol.

MENTION IDEAL FEATURES OF FERMENTOR.WRITE ABOUT ANY TWO TYPES OF FERMENTOR.

- The industrial microorganisms require to grow in a large vessel containing considerable quantities of nutrient media by maintaining favorable conditions. These containers are called "**Fermentors**".

- These fermentors provide optimum growth and metabolism to microbe strains for production of the desired product.

- The term 'bioreactor' is often used synonymously for the fermentor. Generally, fermentors are used for the growth of prokaryotic cells such as bacteria, actinomycetes etc, while bioreactors are used for the growth of eukaryotic cells such as an insect, mammalian cells etc.

- The design of the fermentor depends upon the purpose for which it is to be used.

- Bioreactors arc extensively used for food processing, fermentation, waste treatment, etc.

Ideal features of Fermentor

1. It should provide the best possible growth conditions to industrial strains.

2. The fermentor should be so designed that it may be steam-sterilized under pressure.

3. It must make some provision for the control of contaminating microorganisms during fermentation.

4.It should have the provision and control aver various operations like pH, temperature, agitation, aeration etc it may have facilities for monitoring all conditions.

5.The fermentor should provide all aseptic conditions at the time of sample withdrawal and addition of inoculum.

6.The fermentor vessels must be strong enough to withstand the pressures and toxicity of media.

7.Fermentor must have the facility of incorporation of sterile air and stirring.

8.It should provide all facilities for intermittent addition of antifoam agents, alkali or acid and other nutrients

.

9.The fermentor should have additional inoculum, seed, or media tanks.

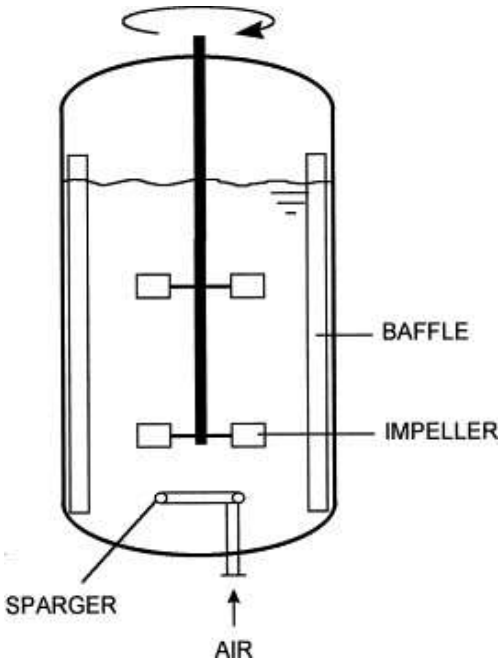
10.It should be designed in such a way that it consumes less power, has less evaporation, can be used for long periods of operation, and has proper sampling facilities.

11.It should have the facility for complete removal of broth from the tank and should be easy to clean.

Types of Fermentors:

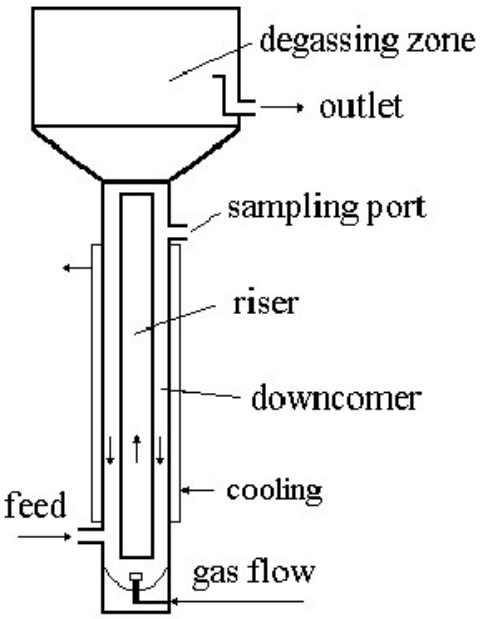
(1) Stirred tank fermenter :

Microbial fermentations are very important in the production of life saving antibiotics. More than 70% fermenters used in the industry are of this type. Stirred tank reactors perform various functions like functions like homogenization, suspension of solids, dispersion of gas-liquid mixtures, aeration of liquid and heat exchange. The Stirred tank reactor is provided with a baffle with a rotating stirrer attached either at the top or at the bottom of the bioreactor. The conventional fermentation is carried out in a batch mode, since stirred tank reactors are easier to operate and cost effective. Many of the industrial bioprocesses even today carried out in batch reactor, since the formation of sub standard product is minimal. The fed batch mode adopted in the recent years eliminates this limitation.

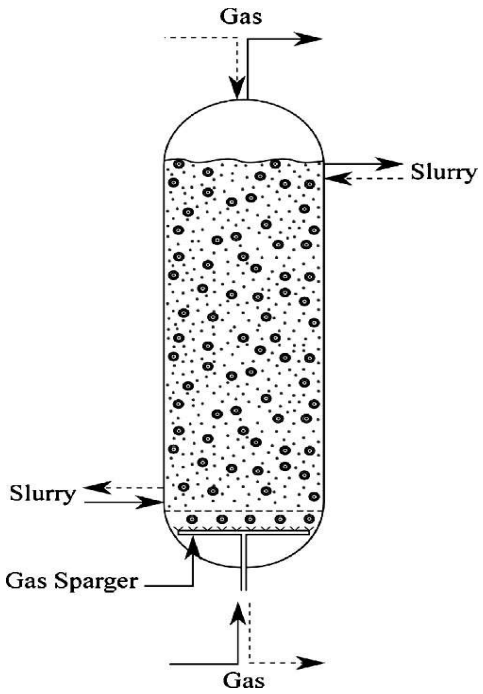


2. Air-Left Fermenter (ALF): This is generally called as pneumatic reactor without any mechanical stirring arrangements for mixing. The turbulence caused by the fluid flow ensures adequate mixing of the liquid. The introduction of the fluid (air/liquid) causes upward motion and results in circulatory flow in the entire reactor. The air/liquid velocities will be low and hence the energy consumption is also low. ALFs can be used for both free and immobilized cells. The advantages of

Airlift reactors are the elimination of attrition effects generally encountered in mechanical agitated reactors. It is ideally suited for aerobic cultures since oxygen mass transfer coefficient are quite high in comparison to stirred tank reactors. This is ideal for SCP production from methanol as carbon substrate. This is used mainly to avoid excess heat produced during mechanical agitation.



3. Bubble Column fermenter: Bubble column fermenter is a simplest type of tower fermenter consisting of a tube which is air sparged at the base. It is an elongated non-mechanically stirred fermenter used for citric acid production.



GIVE AN ACCOUNT OF STEPS INVOLVED IN DOWN STREAM PROCESSING .

- The various procedure involved in the actual recovery of useful products after fermentation or any other process together constitute **Downstream Processing.**
- It is a very important step in the manufacture of different product in pharmaceutical industry (Such as antibiotics, hormones, antibodies, vaccine, enzymes), Food industry etc.
- In addition, the product is either present in the cell, in the medium or both.
- The concentration of product is generally low, in either cases, and it is mixed with other molecules from which it has to be separated.

The various steps of Downstream Processing involve:

1. **Separation**
2. **Cell disruption**
3. **Extraction**
4. **Isolation**
5. **Purification**
6. **Drying**

1.Separation

It is first step of DSP and usually involve the separation of solids substances, from the liquid media.

It is generally achieved by following ways:

- **Filtration**
- **Centrifugation**
- **Flocculation** and **flotation**

Filtration

It is used for filamentous fungi and bacteria.

Different techniques of filtration are as follows:

Surface filtration –

In the surface filtration, also called refuse filtration, the molecules do not penetrate in the filter, but they are retained by its **surface.** The filter pores must be **smaller** than the solid particles to remove.

Depth filtration –

A Depth Filter is a filter consisting of either multiple layers or a single layer of a medium having depth, which captures contaminants within its structure, as opposed to on the surface.

Centrifugal filtration -

Centrifugal filters use the principle of centrifugal force to cause liquid and solids separation. The high centrifugal force pushes the heavy solid particles towards the centrifuge bowl wall while displacing the lighter liquid towards the center of the bowl.

Centrifugation

The technique of centrifugation is based on the principle of density differences between the particles to be separated and the medium. Thus, centrifugation is mostly used for separating solid particles from liquid phase (fluid/particle separation).

In downstream processing,mainly 4 types of centrifuges are used.

- **Tubular bowl centrifuge**
- **Disc centrifuge**
- **Multi-Chamber centrifuge**
- **Scroll centrifuge or decanter**

Flocculation and **flotation**

It is used for small bacterial cells which are difficult to separate even by centrifugation.

Flocculation involves the aggregation of cells which may be induced by inorganic salt, minerals, hydrocolloids and organic polyelectrolytes.If flocculation isn't effective than very minute gas bubbles is made by sparging, release of over pressure or electrolysis.

Flotation,in any case, the gas bubble need to adsorb and surround the cell, raising the gas bubbles to the surface of media in the form of foam.

2.Cell disruption

Disruption of microbial cell is usually difficult because of their small size, rigid cell walls and high osmotic pressure inside the cells.

Disruption of cell is generally achieved by mechanically, lysis or drying:

Mechanical cell disruption:
This involves the uses of shear, E.g.-, colloid mill, ball mill grinder etc., homogenizer and ultrasound.

Lysis:
Lysis of microbial cells may be achieved by chemical means, e.g., salt or surfactants, osmotic shock, freezing, or Lytic enzymes, e.g., lysozyme, etc.

Drying:
It involves the drying of cells by adding the cells into a huge amount of cold acetone and extracted using buffer or salt solution.

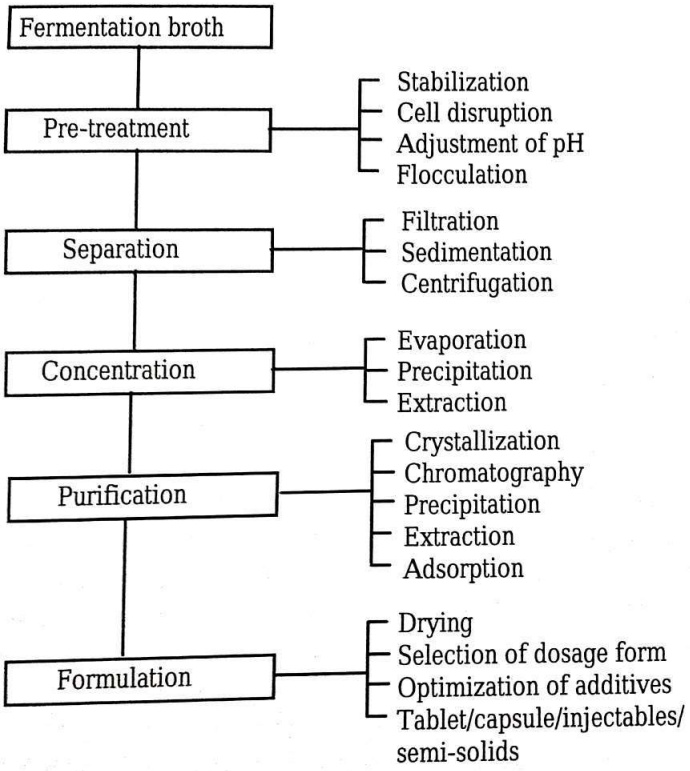
3.Extraction

- It involves the recovery of a compound or a group of compounds from a mixture or from cells into a solvent phase.
- It usually achieved both separation of particles as well as concentration of the product.
- The process of extraction is frequently useful for the recovery of Antibiotics and most of the lipophilic substances.

The process of extraction is achieved by:

Liquid-Liquid Extraction:

Also known as solvent extraction and partitioning, is a method to separate compounds or metal complexes, based on their relative solubilities in two different immiscible liquids, usually water (polar) and an organic solvent (non-polar).



Whole broth (medium + cells) extraction:

The concept of recovering a metabolite directly from an unfiltered fermentation broth is of considerable interest because of its simplicity, the reduction in process stages and the potential cost savings.

Aqueous multiphase extraction:

It is a liquid-liquid fractionation technique and has gained an interest because of great potential for the extraction, separation, purification and enrichment of proteins, membranes, viruses, enzymes, nucleic acids and other biomolecules.

4.Isolation

Some of the product concentration may occur during the extraction step.
It is generally achieved by the following:

Evaporation: E.g.- continuous flow evaporator, falling film evaporator, thin film evaporator, spray dryers.

Membrane filtration:E.g.- Microfiltration, ultrafiltration, reverse osmosis and electrodialysis.

Ion exchange resins:E.g.- Dextran cellulose, polyamine, acrylate etc.

Adsorption resins:
These may be

Polar – Sulfoxide, amide.
Apolar – Styrene-divinyl benzene.
Semipolar – Acrylic ester.

5.Purification

It aims at recovery of the product in a highly purified state.
Purification is achieved by the following procedures:

Crystallization: This is used for the low molecular mass compound like antibiotics.
Chromatographic methods:
It is generally, used for the purification of low molecular mass compound from mixture of similar molecules,
Example: antibiotics(homologous) and macromolecules (enzymes).

The different chromatographic procedures are:

1. Adsorption
2. Ion exchange
3. Gel filtration
4. Hydrophobic
5. Affinity
6. Partition chromatography.

6.Drying

Drying is the most important step in downstream processing which makes the product suitable for handling and storage.
The most frequent approaches of drying are:

Vacuum drying:

Vacuum drying is generally used for the drying of substances which are hygroscopic and heat sensitive, and is based on the principle of creating a vacuum to decrease the chamber pressure below the vapor pressure of the water, causing it to boil.

Spray drying:

Spray drying is a method of producing a dry powder from a liquid by rapidly drying with a hot gas.

Freeze drying:

Freeze drying, also known as lyophilization or cryodesiccation, is a low temperature dehydration process that involves freezing the product, lowering pressure, then removing the ice by sublimation.

Ex:Penicillin can be freeze dried directly in Ampoules.

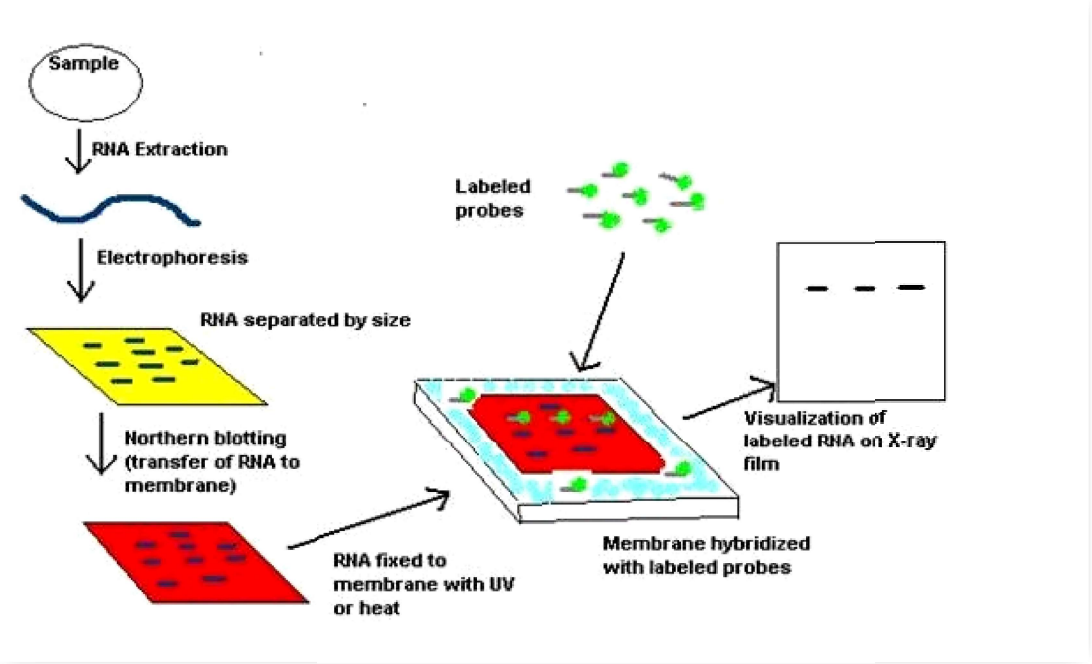
SHORT ANSWER QUESTIONS

NORTHERN BLOTTING TECHNIQUE

Northern blotting technique wasderivedby Alwine and his colleagues in 1979 to separate derived m-RNA from a sample. RNA molecules have defined length & RNA sample are separated based on size by gel electrophoresis .RNA is more susceptible to degradation than DNA .

Much shorter than genomic DNA it is not necessary to cleave RNA before electrophoresis.

- m-RnA is isolated from a cell line at a particular stage and electrophoresed in Agarose gel electrophoresis.
- The electrophorosed gel is immersed in a depurination buffer for 10 minutes and then washed with water.



- The m-RNA in the Agarose gel are transferred to an Aminobenzyoate methyl filter paper.
- The m-RNA blotting filter paper is baked at 800 , the bloted filter is treated with pre-hybridization solution and then placed in aheat resistant bag.
- A particular DNA or RNA probe and hybridization probe are filled in the bag and the bag is sealed.
- The bag is kept at420C for 4-8 hours to establish hybridization.
- The filter is then washed with a wash solution to remove unbounded probes.
- An autoradiogram is taken from filter to know the position of filter having hybrid nucleic acid.

Applications:

Northern blotting helps in studying gene expression pattern of various tissues, organs, developmental stages, pathogen infection, and also over the course of treatment.

It has been employed to study over expression of oncogenes and down-regulation of tumor-suppressor genes in cancerous cells.

Northern blotting is also used for the analysis of alternate spliced products of same gene or repetitive sequence motif by investigating the various sized RNA of the gene.

Variations in size of a gene product may also help to identify deletions or errors in transcript processing, by altering the probe target that can be used along the known sequence and make it possible to determine the missing region of the RNA.

WESTERN BLOTTING TECHNIQUE

A molecular technique of transferring proteins, DNA or RNA, onto a carrier or membrane. Done after a gel electrophoresis, transferring the molecules from the gel onto the blotting membrane or adding the samples directly onto the membrane.

Detect specific proteins in a sample of tissue homogenate or extract using labelled antibodies. The method originated in the laboratory of Harry Towbin at the Friedrich Miescher Institute, Switzerland in 1979. The name western blot was given to the technique by W. Neal Burnette and is a play on the name Southern .

Procedure of western blotting-

Tissue separation:
Samples may be taken from whole tissue or from cell culture. In most cases, solid tissues are first broken down mechanically using a blender. Assorted detergents, salts, and buffers used for lysis of cells and to solubilize proteins.Tissue preparation is often done at cold temperatures to avoid protein denaturing.

Gel Electrophoresis:
The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point, molecular weight, electric charge, or a combination of these factors.

Transferring:

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). The membrane is placed on top of the gel, and a stack of filter papers placed on top of that.The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. Another method for transferring the proteins is called electro blotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane.

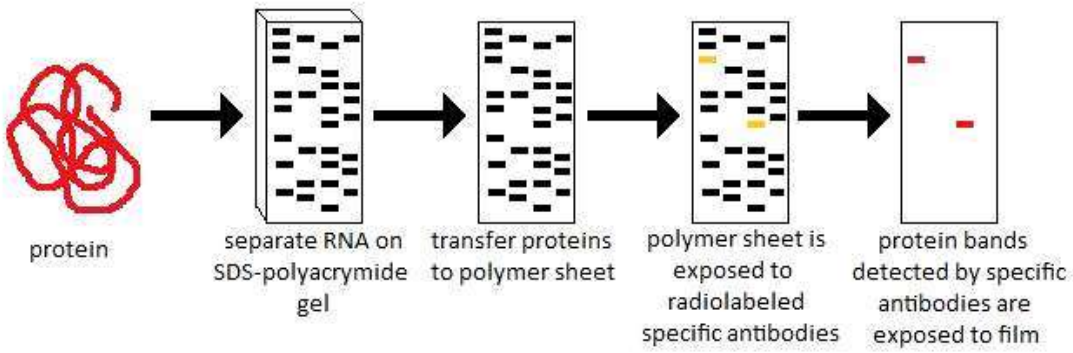
Blocking:
The membrane has the ability to bind to proteins. In this case both the target and antibodies are proteins and so there could be some unwanted binding. Blocking of

non-specific binding is achieved by placing the membrane in a dilute solution of protein typically Bovine serum albumin(BSA) with a minute percentage of detergent such as Tween 20.The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein.

Detection :

Antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. Several secondary antibodies will bind to one primary antibody and enhance the signal.

ANALYSIS:
The colorimetric detection method depends on incubation of the western blot with a substrate that reacts with the reporter enzyme (such as peroxidase).



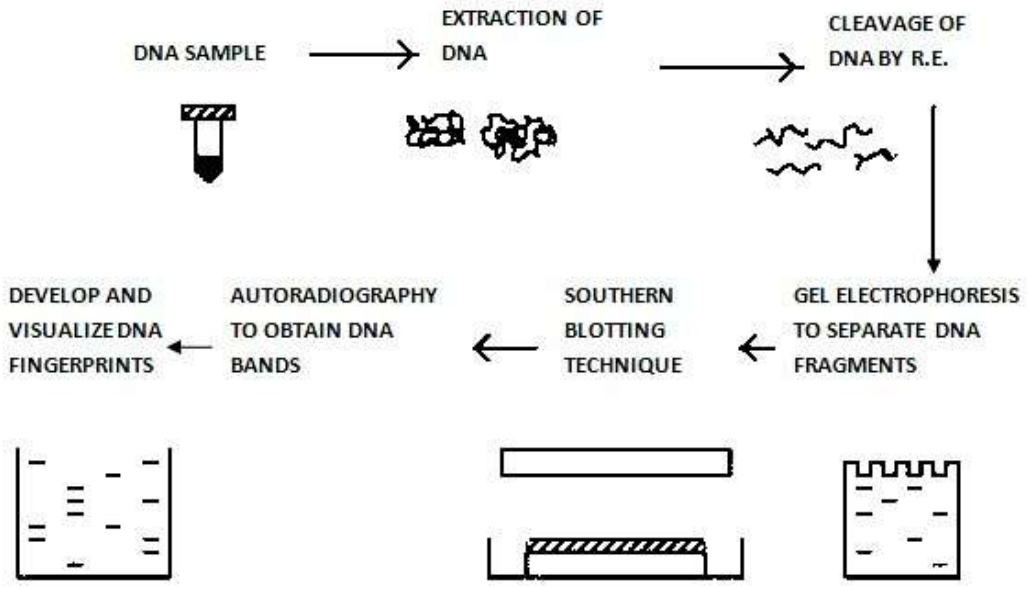
Chemiluminescent detection –
Chemiluminescent detection methods depend on incubation of the western blot with a substrate that will luminescent when exposed to the reporter on the secondary antibody.

Radioactive detection –
Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of Interest.

DNA FINGER PRINTING

It is a test to identify and evaluate the genetic information called DNA (deoxyribonucleic acid)-in a person's cells.
It is called a fingerprint because it is very unlikely that any two people would have exactly the same DNA information and hence the same physical fingerprint.

The test is used to determine whether a family relationship exists between two people, to identify organisms causing a disease, to decide the parentage, to decide the siblings, and to solve crimes, Identify a body which is badly decomposed or if only body parts are available following a natural disaster or a battle.



Only a small sample of cells is needed for DNA fingerprinting. A drop of blood or the root of a hair contains enough DNA for testing. Semen, hair, or skin scrapings,

blood droplets, simple mouthwash or a swab of the cheeks inside the mouth etc., are often used in criminal investigations.

Procedure for DNA finger printing technology:

(i) Collect samples of DNA-containing substance (blood, semen) from suspect caught at the crime scene.

(ii) Amplify quantity using PCR if necessary.

(iii) Add endonucleases to produce DNA RFLPs.

(iv) Use gel electrophoresis to separate RFLPS into DNA fingerprint
.
(v) Compare samples from crime scene and suspect for match.

CONTINUOUS FERMENTATION

Continuous fermentation is an open operation system with continuous addition and discharge of the solution in the system. Microorganisms and sterile nutrient solution are added homogeneously to the bioreactor, continuously, while nutrient solution and microorganisms are transformed equivalently in the system.

- Here the exponential growth rate of the microbes is maintained in the fermentor for prolonged periods of time in by the addition of fresh media are regular intervals.
- Microbes reach the exponential growth rate and continue as such due to the availability of nutrients.The microbes go through a very short lag phase and then maintain themselves at the exponential phase.
- The exponential growth rate of microbes continues till the vessel becomes completely filled in the cells.
- Continuous fermentor possesses devices for the collection of overflow from the vessel.
- The metabolite or the product of fermentation is extracted for the overflow by downstream processing.

- Thus unlike batch fermentation, in continuous fermentation, the fermentation process never stops in between and it continues to run for a long period of time with the addition of nutrients and harvesting the metabolites at regular intervals.
- Nutrients are added throughout the batch. Nutrients are consumed rapidly.
- The product is continuously removed while the batch is operating
- The turnover rate is high.
- It is used to extract primary metabolites such as amino acids and proteins.
- The growth conditions have to be maintained throughout the operation of the batch.
- Small size fermenters are used. It has a high chance of contamination.

MONOCULTURE IN FISHES

Monoculture can be defined as the culture of a single species of an organism in a fresh, brackish or salt water culture systems to marketable size.

Objectives of Monoculture :

(1) To get maximum amount of production, and to prevent species extinction by over exploitation.

(2) High nutrient rich fish cultivation

(3) Supply high quality animal protein and vitamin rich oil like cod liver fish oil from cod fish.

Cultivable fish species in monoculture should

1. have fast growth rate.
2. have ability to feed on natural and cheap artificial food.
3. be strong and resistant to diseases.
4. be able to tolerate adverse and physico-chemical conditions of pond water.

5. consume small quantity of food for growth and development.

6. possess high nutritive value.

7.be able to reproduce under confined conditions

8.Support high population density in pond

9.Specific feed is important and form main basis for monoculture in fishes.

Advantages :

- Monoculture fish farming is easy to monitor individual fish breed performance.
- There is no undue competition for space and feed.

Disadvantages:

Regression in water quality, cannibalism(eating of their own species), disease outbreak due to overstocking of pond with fish.

Factors to be considered while establishing the monoculture fish farm;

1. Availability of good quality water and fish feed.
2. Availability of suitable fingerling growing rapidly and have good feed to flesh convertibility.
3. Good Soil type(clay soil).
4. Avoid such soils having heavy metals like copper, mercury, arsenic and lead.
5. Avoid highly industrialized area as the sulphur fumes released are poisonous to fish.
6. Vegetation of the soil must be easy to clear.
7. Topography of the land must not be easily flooded.

Indigenous fishes selected in monoculture :

(a)Natural fresh water fishes: Mesocarps like Catla catla, Labeo rohita, Cirrihinus mrigala, Labeo calbasu, Cirrihinus cirrhosa.

(b)Brackish water fishes: Milkfish,(Chanos chanos) and Mullets in several countries.

(c)Salt water species Exotic carps : Hypothalmichthys molitrix (silver carp), Ctenopharyngodon idella (grass carp), Cyprinus carpio (common carp), Eel (Anguilla spp) in several countries.

POLYPLOIDY IN FISHES

Polyploidy is the condition of possessing more than two complete sets of chromosomes.
Fishes are characterized by great diversity in ecology, morphology, life history, behavior and physiology.

Most fish are diploid (2N).~50 chromosomes is the typical number of chromosomes seen in fishes.Normally, they Produce haploid (1N) gametes. Duplication of entire chromosome set due to the failure of meiosis result in the formation of diploid gametes. This occurs mostly in female fishes.

Fertilization of such a diploid egg by a normal spermatozoan or the fertilization of the normal egg by a diploid spermatozoan leads to the appearance of triploid viable organisms.

Ex: Carassius auratus gibelio and Poeciliopsis sp., Poecilia sp., and rainbow trout are triploid.

Tetraploid fishes also appear as a result of fusion of the male and femalediploid gametes. There are no data on the frequency of such mutations in nature. Such triploid or tetraploid fishes are also called as polyploid organisms.

Polyploidy may be of two types viz.,

(i) Autopolyploidy as seen in salmonella(trout fish), embryo parents of the same species andis derived from parents of the same species

(ii) Allopolyploidy, embryo is formed from parents of different species as seen in catostomid group of fishes.

Misgurnus anguillicaudatus (Oriental loach) is the naturally occurring polyploid fish having 50 chromosomes in diploid, 75 chromosomes in triploid and 100 chromosomes in tetraploid condition.

Polyploidy in fishes can be attributed as a consequence of distant hybridization.