IMMUNOLOGY AND ANIMAL BIOTECHNOLOGY

UNIT- 1

1.1. BASIC CONCEPTS OF IMMUNOLOGY:

The **term** immunology was coined by Russian biologist **Ilya Ilyich Mechnikov**, who advanced studies on **immunology** and received the Nobel Prize for his work in 1908. He pinned small thorns into starfish larvae and noticed unusual cells surrounding the thorns. This was the active response of the body trying to maintain its integrity. **Basic Immunology** focuses on substances that take part in serological reactions, including antigens, antibodies, and the physicochemical nature of **immunological** reactions. The selection first elaborates on the **basic** notions of immunity, antigens, immunoglobulins, and the production of antibody. Edward Jenner was the first scientist, who paved the discovery of vaccine against the cowpox (1796). This attempt induced protection against human small pox.

When Jenner introduced vaccination he knew nothing of the infectious agents that cause disease: it was not until late in the 19th century that Robert Koch proved that infectious diseases are caused by microorganisms, each one responsible for a particular disease, or pathology. We now recognize four broad categories of disease-causing microorganisms, or **pathogens**: these are viruses, bacteria, pathogenic fungi, and other relatively large and complex eukaryotic organisms collectively termed parasites.

The discoveries of Koch and other great 19th century microbiologists stimulated the extension of Jenner's strategy of vaccination to other diseases. In the 1880s, Louis Pasteur devised a vaccine against cholera in chickens, and developed a rabies vaccine that proved a spectacular success upon its first trial in a boy bitten by a rabid dog. These practical triumphs led to a search for the mechanism of protection and to the development of the science of immunology. In 1890, Emil von Behring and Shibasaburo Kitasato discovered that the serum of vaccinated individuals contained substances—which they called **antibodies**—that specifically bound to the relevant pathogen.

A specific immune response, such as the production of antibodies against a particular pathogen, is known as an adaptive immune response, because it occurs during the lifetime of an individual as an adaptation to infection with that pathogen. In many cases, an adaptive immune response confers lifelong protective immunity to reinfection with the same pathogen. This distinguishes such responses from innate immunity, which, at the time that von Behring and Kitasato discovered antibodies, was known chiefly through the work of the great Russian immunologist Elie Metchnikoff. Metchnikoff discovered that many microorganisms could be engulfed and digested by **phagocytic cells**, which he called macrophages. These cells are immediately available to combat a wide range of pathogens without requiring prior exposure and are a key component of the innate immune system. Antibodies, by contrast, are produced only after infection, and are specific for the infecting pathogen. The antibodies present in a given person therefore directly reflect the infections to which he or she has been exposed.

Indeed, it quickly became clear that specific antibodies can be induced against a vast range of substances. Such substances are known as antigens because they can stimulate the *gen*eration of *anti*bodies. We shall see, however, that not all adaptive immune responses entail the

production of antibodies, and the term antigen is now used in a broader sense to describe any substance that can be recognized by the adaptive immune system.

Both innate immunity and adaptive immune responses depend upon the activities of white blood cells, or leukocytes. Innate immunity largely involves granulocytes and macrophages. Granulocytes, also called polymorphonuclear leukocytes, are a diverse collection of white blood cells whose prominent granules give them their characteristic staining patterns; they include the neutrophils, which are phagocytic. The macrophages of humans and other vertebrates are presumed to be the direct evolutionary descendants of the phagocytic cells present in simpler animals, such as those that Metchnikoff observed in sea stars. Adaptive immune responses depend upon lymphocytes, which provide the lifelong immunity that can follow exposure to disease or vaccination. The innate and adaptive immune systems together provide a remarkably effective defense system. It ensures that although we spend our lives surrounded by potentially pathogenic microorganisms, we become ill only relatively rarely. Many infections are handled successfully by the innate immune system and cause no disease; others that cannot be resolved by innate immunity trigger adaptive immunity and are then overcome successfully, followed by lasting immunological memory.

In simple words we can say that immuology is the study of immune system of vertebrates, which involves the resistance shown and protection offered by the host organism against the infectious diseases. The immunes ystemc onsistso f a complex network of cells and molecules, and their interactions.It is specifically designed to eliminate infectious organisms from the body. This is possible since the organism is capable of distinguishing the self from non-self, and eliminate non-self. Immunity is broadly divided into two types - innate (non-specific)im munity and adaptive or acqui red(speci f ic)im muni ty.

INNATE IMMUNITY

Innate immunity is non-specific, and represents the inherent capability of the organism to offer resistance against diseases. It consists of defensive barriers.

First line of defense. The skin is the largest organ in the human body, constituting about 15% of the adult body weight .The skin provides mechanical barrier to preventt he entry of microorganisms and viruses. The acidic (pH 3-5) environment on the skin surface inhibits the growth of certain. Microorganisms. Further, sweat contains an enzyme lysozyme that can destroy bacterial cell wall**.**

Second line of defense. Despite the physical barriers, the microorganisms do enter the body. The body defends itself and eliminates the invading organisms by non-specific mechanisms such as sneezing and secretions of the mucus. In addition, the body also tries to kill the pathogens by phagocytosis (involving macrophages and complement system). The inflammatory response and fever responseo f the body also form a part of innate immunity.

ADAPTIVE IMMUNITY

Adaptive immunity is capable of recognizing and selectively eliminating specific foreign microorganisms and molecules (i.e., foreign antigens). Unlike innate immune responses, adaptive immune responses are not the same in all members of a species but are reactions to specific antigenic challenges.Adaptive immunity displays four characteristic attributes: _

- Antigenic specificity

- Diversity
- Immunologic memory
- Self/nonself recognition

A. CELLS OF IMMUNE SYSTEM:

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

LYMPHOID CELLS:

Lymphocytes constitute 20%–40% of the body's white blood cells and 99% of the cells in the lymph. lymphocytes in the human body. These lymphocytes continually circulate in the blood and lymph and are capable of migrating into the tissue spaces and lymphoid organs, thereby integrating the immune system to a high degree. The lymphocytes can be broadly subdivided into three populations—B cells, T cells, and natural killer cells—on the basis of function and cell-membrane components. **Natural killer cells (NK cells)** are large, granular lymphocytes that do not express the set of surface markers typical of B or T cells. Resting B and T lymphocytes are small, motile, nonphagocytic cells,which cannot be distinguished morphologically. Lymphoblasts proliferate and eventually differentiate into **effector cells** or into **memory cells.** Effector cells function in various ways to eliminate antigen. These cells have short life spans, generally ranging from a few days to a few weeks. **Plasma cells**—the antibody-secreting effector cells of the Bcell lineage—have a characteristic cytoplasm that contains abundant endoplasmic reticulum (to support their high rate of protein synthesis) arranged in concentric layers and also many Golgi vesicles. The effector cells of the T-cell lineage include the cytokine-secreting T helper cell (TH cell) and the T cytotoxic lymphocyte (TC cell). Some of the progeny of B and T lymphoblasts differentiate into memory cells. The persistence of this population of cells is responsible for life-long immunity to many pathogens. Memory cells look like small lymphocytes but can be distinguished from naive cells by the presence or absence of certain cellmembrane molecules.

B LYMPHOCYTES

The B lymphocyte derived its letter designation from its site of maturation, in the *b*ursa of Fabricius in birds; the name turned out to be apt, for *b*one marrow is its major site of maturation in a number of mammalian species, including humans and mice. Mature B cells are definitively distinguished from other lymphocytes by their synthesis and display of membrane-bound immunoglobulin (antibody) molecules, which serve as receptors for antigen. Interaction between antigen and the membrane-bound antibody on a mature naive B cell, as well as interactions with T cells and macrophages, selectively induces the activation and differentiation of B-cell clones of corresponding specificity. In this process, the B cell divides repeatedly and differentiates over a 4- to 5-day period, generating a population of plasma cells and memory cells. Plasma cells, which have lower levels of membrane-bound antibody than B cells, synthesize and secrete antibody. All clonal progeny from a given B cell secrete antibody molecules with the same antigen-binding specificity. Plasma cells are terminally differentiated cells, and many die in 1 or 2 weeks.

T LYMPHOCYTES

T lymphocytes derive their name from their site of maturation in the thymus. Like B lymphocytes, these cells have membrane receptors for antigen. Although the antigenbinding T-cell receptor is structurally distinct from immunoglobulin, it does share some common structural features with the immunoglobulin molecule, most notably in the structure of its antigen-binding site. Unlike the membrane- bound antibody on B cells, though, the T-cell receptor (TCR) does not recognize free antigen. Instead the TCR recognizes only antigen that is bound to particular classes of self-molecules.

Most T cells recognize antigen only when it is bound to a self-molecule encoded by genes within the major histocompatibility complex (MHC), a fundamental difference between the humoral and cell-mediated branches of the immune system is that the B cell is capable of binding soluble antigen,whereas the T cell is restricted to binding antigen displayed on self-cells. To be recognized by most T cells, this antigen must be displayed together with MHC molecules on the surface of antigen-presenting cells or on virus-infected cells, cancer cells, and grafts. The T-cell system has developed to eliminate these altered self-cells,which pose a threat to the normal functioning of the body. Like B cells, T cells express distinctive membrane molecules. All T-cell subpopulations express the T-cell receptor, a complex of polypeptides that includes CD3; and most can be distinguished by the presence of one or the other of two membrane molecules, CD4 and CD8. In addition, most mature T cells express the following membrane molecules.

T cell. In general, expression of CD4 and of CD8 also defines two major functional subpopulations of T lymphocytes. CD4+ T cells generally function as T helper (TH) cells and are class-II restricted; CD8+ T cells generally function as T cytotoxic (TC) cells and are class-I restricted. Thus the ratio of TH to TC cells in a sample can be approximated by assaying the number of CD4+ and CD8+ T cells. This ratio is approximately 2:1 in normal human peripheral blood, but it may be significantly altered by immunodeficiency diseases, autoimmune diseases, and other disorders. The classification of CD4+ class II–restricted cells as TH cells and CD8+ class I–restricted cells as TC cells is not absolute. Some CD4+ cells can act as killer cells. Also, some TC cells have been shown to secrete a variety of cytokines and exert an effect on other cells comparable to that exerted by TH cells.

The distinction between TH and TC cells, then, is not alwaysclear; there can be ambiguous functional activities.m However, because these ambiguities are the exception and not the rule, the generalization of T helper (TH) cells as being CD4+ and class-II restricted and of T cytotoxic cells (TC) as being CD8+ and class-I restricted is assumed throughout this text, unless otherwise specified. TH cells are activated by recognition of an antigen–class II MHC complex on an antigen-presenting cell. After activation, the TH cell begins to divide and gives rise to a clone of effector cells, each specific for the same antigen–class II MHC complex.

These TH cells secrete various cytokines, which play a central role in the activation of B cells, T cells, and other cells that participate in the immune response. Changes in the pattern of cytokines produced by TH cells can change the type of immune response that develops among other leukocytes.

The **TH1 response** produces a cytokine profile that supports inflammation and activates mainly certain T cells and macrophages, whereas the **TH2 response** activates mainly B cells and immune responses that depend upon antibodies. TC cells are activated when they interact with an antigen–class I MHC complex on the surface of an altered self-cell (e.g., a virus-infected cell or a tumor cell) in the presence of appropriate cytokines. This activation, which results in proliferation, causes the TC cell to differentiate into

an effector cell called a **cytotoxic T lymphocyte (CTL).** In contrast to TH cells, most CTLs secrete few cytokines. Instead, CTLs acquire the ability to recognize and eliminate altered self-cells.

Another subpopulation of T lymphocytes—called **T suppressor (TS) cells**—has been postulated. It is clear that some T cells help to suppress the humoral and the cell-mediated branches of the immune system, but the actual isolation and cloning of normal TS cells is a matter of controversy and dispute among immunologists. For this reason, it is uncertain whether TS cells do indeed constitute a separate functional subpopulation of T cells. Some immunologists believe that the suppression mediated by T cells observed in some systems is simply the consequence of activities of TH or TC subpopulations whose end results are suppressive.

NATURAL KILLER CELLS

The natural killer cell was first described in 1976,when it was shown that the body contains a small population of large, granular lymphocytes that display cytotoxic activity against a wide range of tumor cells in the absence of any previous immunization with the tumor. NK cells were subsequently shown to play an important role in host defense both against tumor cells and against cells infected with some, though not all, viruses. These cells, which constitute 5%–10% of lymphocytes in human peripheral blood, do not express the membrane molecules and receptors that distinguish T- and B-cell lineages.

Although NK cells do not have T-cell receptors or immunoglobulin incorporated in their plasma membranes, they can recognize potential target cells in two different ways. In some cases, an NK cell employs NK cell receptors to distinguish abnormalities, notably a reduction in the display of class I MHC molecules and the unusual profile of surface antigens displayed by some tumor cells and cells infected by some viruses.

Another way in which NK cells recognize potential target cells depends upon the fact that some tumor cells and cells infected by certain viruses display antigens against which the immune system has made an antibody response, so that antitumor or antiviral antibodies are bound to their surfaces.

Mononuclear Phagocytes

The mononuclear phagocytic system consists of **monocytes** circulating in the blood and **macrophages** in the tissues. During hematopoiesis in the bone marrow, granulocyte-monocyte progenitor cells differentiate into promonocytes, which leave the bone marrow and enter the blood, where they further differentiate into mature monocytes. Monocytes circulate in the bloodstream for about 8 h, during which they enlarge; they then migrate into the tissues and differentiate into specific tissue macrophages or, as discussed later, into dendritic cells.

Differentiation of a monocyte into a tissue macrophage involves a number of changes: The cell enlarges five- to tenfold; its intracellular organelles increase in both number and complexity; and it acquires increased phagocytic ability, produces higher levels of hydrolytic enzymes, and begins to secrete a variety of soluble factors. Macrophages are dispersed throughout the body. Some take up residence in particular tissues, becoming fixed macrophages, whereas others remain motile and are called free, or wandering, macrophages. Free macrophages travel by amoeboid movement throughout the tissues.Macrophage-like cells serve different functions in different tissues and are named according to their tissue location:

- **Alveolar macrophages** in the lung

- **Histiocytes** in connective tissues
- **Kupffer cells** in the liver
- **Mesangial cells** in the kidney
- **Microglial cells** in the brain
- **Osteoclasts** in bone

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

PHAGOCYTOSIS

Macrophages are capable of ingesting and digesting exogenous antigens, such as whole microorganisms and insoluble particles, and endogenous matter, such as injured or dead host cells, cellular debris, and activated clotting factors. In the first step in phagocytosis, macrophages are attracted by and move toward a variety of substances generated in an immune response; this process is called **chemotaxis.** The next step in phagocytosis is adherence of the antigen to the macrophage cell membrane.
Complex antigens, such as whole bacterial cells or viral particles, tend to adhere well and are readily phagocytosed; isolated proteins and encapsulated bacteria tend to adhere poorly and are less readily phagocytosed. Adherence induces membrane protrusions, called **pseudopodia,** to extend around the attached material.
Fusion of the pseudopodia encloses the material within a membrane-bounded structure called a **phagosome,** which then enters the endocytic processing pathway. In this pathway, a phagosome moves toward the cell interior, where it fuses with a **lysosome** to form a **phagolysosome.** Lysosomes contain lysozyme and a variety of other hydrolytic enzymes that digest the ingested material. The digested contents of the phagolysosome are then eliminated in a process called **exocytosis.**

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

NEUTROPHILS

Neutrophils are produced by hematopoiesis in the bone marrow. They are released into the peripheral blood and circulate for 7–10 h before migrating into the tissues, where they have a life span of only a few days. In response to many types of infections, the bone marrow releases more than the usual number of neutrophils and these cells generally are the first to arrive at a site of inflammation. The resulting transient increase
in the number of circulating neutrophils, called **leukocytosis,** is used medically as an indication of infection. Movement of circulating neutrophils into tissues, called **extravasation,** takes several steps: the cell first adheres to
the vascular endothelium, then penetrates the gap between adjacent endothelial cells lining the vessel wall, and finally penetrates the vascular basement membrane, moving out into the tissue spaces. A number of substances generated in an inflammatory reaction serve as **chemotactic factors** that promote accumulation of neutrophils at an inflammatory site.Among these chemotactic factors are some of the complement components, components of the blood-clotting system, and several cytokines secreted by activated TH cells and macrophages.

EOSINOPHILS

Eosinophils, like neutrophils, are motile phagocytic cells that can migrate from the blood into the tissue spaces. Their phagocytic role is significantly less important than that of neutrophils, and it is thought that they play a role in the defense against parasitic organisms. The secreted contents of eosinophilic granules may damage the parasite membrane. BASOPHILS Basophils are nonphagocytic granulocytes that function by releasing pharmacologically active substances from their cytoplasmic granules. These substances play a major role in certain allergic responses.

MAST CELLS

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

DENDRITIC CELLS

The **dendritic cell (DC)** acquired its name because it is covered with long membrane extensions that resemble the dendrites of nerve cells. Dendritic cells can be difficult to isolate because the conventional procedures for cell isolation tend to damage their long extensions. The development of isolation techniques that employ enzymes and gentler dispersion has facilitated isolation of these cells for study in vitro. There are many types of dendritic cells, although most mature dendritic cells have the same major function, the presentation of antigen to TH cells. Four types of dendritic cells are known: Langerhans cells, interstitial dendritic cells, myeloid cells, and lymphoid dendritic cells.

B. Organs of immune system – primary and secondary lymphoid organs:

PRIMARY LYMPHOID ORGANS

A number of morphologically and functionally diverse organs and tissues have various functions in the development of immune responses. These can be distinguished by function as the **primary** and **secondary lymphoid organs** . The thymus and bone marrow are the primary (or central) lymphoid organs, where maturation of lymphocytes takes place. The lymph nodes, spleen, and various mucosalassociated lymphoid tissues (MALT) such as gut-associated lymphoid tissue (GALT) are the secondary (or peripheral) lymphoid organs, which trap antigen and provide sites for mature lymphocytes to interact with that antigen. In addition, **tertiary lymphoid tissues,** which normally contain fewer lymphoid cells than secondary lymphoid organs, can import lymphoid cells during an inflammatory response. Most prominent of these are cutaneous-associated lymphoid tissues. Once mature lymphocytes have been generated in the primary lymphoid organs, they circulate in the blood and **lymphatic system,** a network of vessels that collect fluid that has escaped into the tissues from capillaries of the circulatorysystem and ultimately return it to the blood.

Thymus:

The thymus increases in size from birth in response to postnatal antigen stimulation. It is most active during the neonatal and pre-adolescent periods. At puberty, by the early teens, the thymus begins to atrophy and regress, with adipose tissue mostly replacing the thymic stroma. However, residual T lymphopoiesis continues throughout adult life. The loss or lack of the thymus results in severe immunodeficiency and subsequent high susceptibility to infection. In most species, the thymus consists of lobules divided by septa which are made up of epithelium; it is therefore often considered an epithelial organ. T cells mature from thymocytes, proliferate, and undergo a selection process in the thymic cortex before entering the medulla to interact with epithelial cells.

The thymus provides an inductive environment for the development of T cells from hematopoietic progenitor cells. In addition, thymic stromal cells allow for the selection of a functional and self-tolerant T cell repertoire. Therefore, one of the most important roles of the thymus is the induction of central tolerance.

The function of the thymus is to generate and select a repertoire of T cells that will protect the body from infection.As thymocytes develop, an enormous diversity of T-cell receptors is generated by a random process (see Chapter 9) that produces some T cells with receptors capable of recognizing antigen-MHC complexes.However, most of the T-cell receptors produced by this random process are incapable of recognizing antigen-MHC complexes and a small portion react with combinations of self antigen-MHC complexes. Using mechanisms that are discussed in Chapter 10, the thymus induces the death of those T cells that cannot recognize antigen- MHC complexes and those that react with self-antigen– MHC and pose a danger of causing autoimmune disease. More than 95% of all thymocytes die by apoptosis in the thymus without ever reaching maturity.

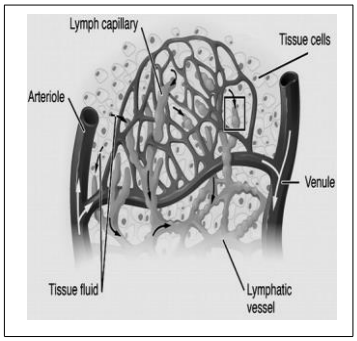
Bone marrow:

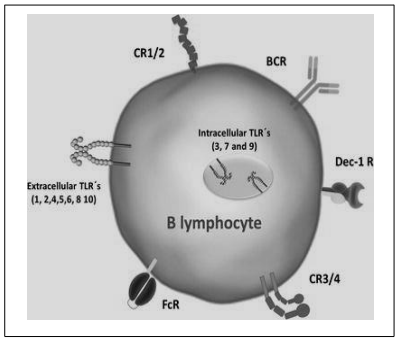
Bone marrow is responsible for both the creation of T cells and the production and maturation of B cells, which are important cell types of the immune system. From the bone marrow, B cells immediately join the circulatory system and travel to secondary lymphoid organs in search of pathogens. T cells, on the other hand, travel from the bone marrow to the thymus, where they develop further and mature. Mature T cells then join B cells in search of pathogens. The other

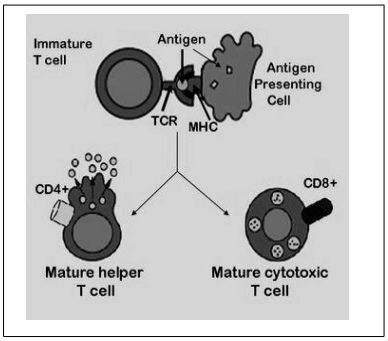
95% of T cells begin a process of apoptosis, a form of programmed cell death. In birds, a lymphoid organ called the bursa of Fabricius, a lymphoid tissue associated with the gut, is the primary site of B-cell
maturation. In mammals such as primates and rodents, there is no bursa and no single counterpart to it as a primary lymphoid organ. In cattle and sheep, the primary lymphoid tissue hosting the maturation, proliferation, and diversification of B cells early in gestation is the fetal spleen.

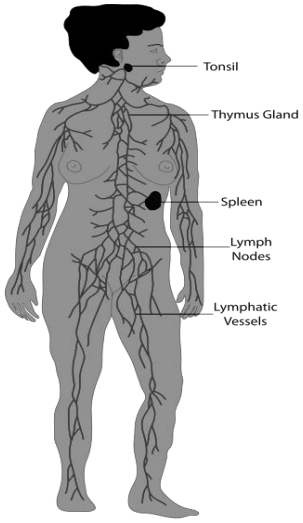
LYMPHATIC SYSTEM:

(plasma) seeps through the thin wall of the capillaries into the surrounding tissue.Much of this fluid, called **interstitial fluid,** returns to the blood through the capillary membranes. The remainder of the interstitial fluid, now called **lymph,** flows from the spaces in connective tissue into a network of tiny open lymphatic capillaries and then into a series of progressively larger collecting vessels called **lymphatic vessels.** The largest lymphatic vessel, the **thoracic duct,** empties into the left subclavian vein near the heart. The heart does not pump the lymph through the lymphatic system; instead the flow of lymph is achieved as the lymph vessels are squeezed by movements of the body's muscles. A series of one-way valves along the lymphatic vessels ensures that lymph flows only in one direction. When a foreign antigen gains entrance to the tissues, it is picked up by the lymphatic system (which drains all the tissues of the body) and is carried to various organized lymphoid tissues such as lymph nodes, which trap the foreign antigen. As lymph passes from the tissues to lymphatic vessels, it becomes progressively enriched in lymphocytes. Thus, the lymphatic system also serves as a means of transporting lymphocytes and antigen from the connective tissues to organized lymphoid tissues where the lymphocytes may interact with the trapped antigen and undergo activation.









SECONDARY LYMPHOID ORGANS

Various types of organized lymphoid tissues are located along the vessels of the lymphatic system. Some lymphoid tissue in the lung and lamina propria of the intestinal wall consists of diffuse collections of lymphocytes and macrophages.

Other lymphoid tissue is organized into structures called lymphoid follicles, which consist of aggregates of lymphoid and nonlymphoid cells surrounded by a network of draining lymphatic capillaries. Until it is activated by antigen, a lymphoid follicle—called a **primary follicle** —comprises a network of follicular dendritic cells and small resting B

cells. After an antigenic challenge, a primary follicle becomes a larger **secondary follicle**—a ring of concentrically packed B lymphocytes surrounding a center (the **germinal center**) in which one finds a focus of proliferating B lymphocytes and an area that contains nondividing B cells, and some
helper T cells interspersed with macrophages and follicular dendritic cells.

SPLEEN:

The spleen plays a major role in mounting immune responses to antigens in the blood stream. It is a large, ovoid secondary lymphoid organ situated high in the left abdominal cavity. While lymph nodes are specialized for trapping antigen from local tissues, the spleen specializes in filtering blood and trapping blood-borne antigens; thus, it can respond to systemic infections. Unlike the lymph nodes, the spleen is not supplied by lymphatic vessels. Instead, bloodborne antigens and lymphocytes are carried into the spleen through the splenic artery. Experiments with radioactively labeled lymphocytes show that more recirculating lymphocytes pass daily through the spleen than through all the lymph nodes combined.

The main functions of the spleen are:

1. to produce immune cells to fight antigens
2. to remove particulate matter and aged blood cells, mainly red blood cells
3. to produce blood cells during fetal life.

The spleen synthesizes antibodies in its white pulp and removes antibody-coated bacteria and antibody-coated blood cells by way of blood and lymph node circulation. A study published in 2009 using mice found that the spleen contains, in its reserve, half of the body's monocytes within the red pulp. These monocytes, upon moving to injured tissue (such as the heart), turn into dendritic cells and macrophages while promoting tissue healing. The spleen is a center of activity of the mononuclear phagocyte system and can be considered analogous to a large lymph node, as its absence causes a predisposition to certain infections.

Like the thymus, the spleen has only efferent lymphatic vessels. Both the short gastric arteries and the splenic artery supply it with blood. The germinal centers are supplied by arterioles called *penicilliary radicles*.

Until the fifth month of prenatal development, the spleen creates red blood cells; after birth, the bone marrow is solely responsible for hematopoiesis. As a major lymphoid organ and a central player in the reticuloendothelial system, the spleen retains the ability to produce lymphocytes. The spleen stores red blood cells and lymphocytes. It can store enough blood cells to help in an emergency. Up to 25% of lymphocytes can be stored at any one time.

LYMPH NODES:

A lymph node is an organized collection of lymphoid tissue, through which the lymph passes on its way back to the blood. Lymph nodes are located at intervals along the lymphatic system. Several afferent lymph vessels bring in lymph, which percolates through the substance of the lymph node, and is then drained out by an efferent lymph vessel. Of the nearly 800 lymph nodes in the human body, about 300 are located in the head and neck. Many are grouped in clusters in different regions, as in the underarm and abdominal areas. Lymph node clusters are commonly found at the proximal ends of limbs (groin, armpits) and in the neck, where lymph is collected from regions of the body likely to sustain pathogen contamination from injuries. Lymph nodes

are particularly numerous in the mediastinum in the chest, neck, pelvis, axilla, inguinal region, and in association with the blood vessels of the intestines.

The substance of a lymph node consists of lymphoid follicles in an outer portion called the cortex. The inner portion of the node is called the medulla, which is surrounded by the cortex on all sides except for a portion known as the hilum. The hilum presents as a depression on the surface of the lymph node, causing the otherwise spherical lymph node to be bean-shaped or ovoid. The efferent lymph vessel directly emerges from the lymph node at the hilum. The arteries and veins supplying the lymph node with blood enter and exit through the hilum. The region of the lymph node called the paracortex immediately surrounds the medulla. Unlike the cortex, which has mostly immature T cells, or thymocytes, the paracortex has a mixture of immature and mature T cells. Lymphocytes enter the lymph nodes through specialised high endothelial venules found in the paracortex.

A lymph follicle is a dense collection of lymphocytes, the number, size, and configuration of which change in accordance with the functional state of the lymph node. For example, the follicles expand significantly when encountering a foreign antigen. The selection of B cells, or *B lymphocytes*, occurs in the germinal centre of the lymph nodes.

Secondary lymphoid tissue provides the environment for the foreign or altered native molecules (antigens) to interact with the lymphocytes. It is exemplified by the lymph nodes, and the lymphoid follicles in tonsils, Peyer's patches, spleen, adenoids, skin, etc. that are associated with the mucosa-associated lymphoid tissue (MALT).

In the gastrointestinal wall, the appendix has mucosa resembling that of the colon, but here it is heavily infiltrated with lymphocytes.

1.2. INNATE AND ACQUIRED IMMUNITY:

A. INNATE IMMUNITY:

The **innate immune system** is one of the two main immunity strategies found in vertebrates (the other being the adaptive immune system). The innate immune system is an older evolutionary defense strategy, relatively speaking, and is the dominant immune system response found in plants, fungi, insects, and primitive multicellular organisms.

The major functions of the vertebrate innate immune system include:

- Recruiting immune cells to sites of infection through the production of chemical factors, including specialized chemical mediators called cytokines
- Activation of the complement cascade to identify bacteria, activate cells, and promote clearance of antibody complexes or dead cells
- Identification and removal of foreign substances present in organs, tissues, blood and lymph, by specialized white blood cells
- Activation of the adaptive immune system through a process known as antigen presentation
- Acting as a physical and chemical barrier to infectious agents; via physical measures like skin or tree bark and chemical measures like clotting factors in blood or sap from a tree, which are released following a contusion or other injury that breaks through the first-line

physical barrier (not to be confused with a second-line physical or chemical barrier, such as the blood-brain barrier, which protects the extremely vital and highly sensitive nervous system from pathogens that have already gained access to the host's body).

Anatomical barriers include physical, chemical and biological barriers. The epithelial surfaces form a physical barrier that is impermeable to most infectious agents, acting as the first line of defense against invading organisms. Desquamation (shedding) of skin epithelium also helps remove bacteria and other infectious agents that have adhered to the epithelial surfaces. Lack of blood vessels and inability of the epidermis to retain moisture, presence of sebaceous glands in the dermis provides an environment unsuitable for the survival of microbes.[2] In the gastrointestinal and respiratory tract, movement due to peristalsis or cilia, respectively, helps remove infectious agents. Also, mucus traps infectious agents. The gut flora can prevent the colonization of pathogenic bacteria by secreting toxic substances or by competing with pathogenic bacteria for nutrients or attachment to cell surfaces. The flushing action of tears and saliva helps prevent infection of the eyes and mouth.

Inflammation is one of the first responses of the immune system to infection or irritation. Inflammation is stimulated by chemical factors released by injured cells and serves to establish a physical barrier against the spread of infection, and to promote healing of any damaged tissue following the clearance of pathogens.

The process of acute inflammation is initiated by cells already present in all tissues, mainly resident macrophages, dendritic cells, histiocytes, Kupffer cells, and mast cells. These cells present receptors contained on the surface or within the cell, named *pattern recognition receptors* (PRRs), which recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). At the onset of an infection, burn, or other injuries, these cells undergo activation (one of their PRRs recognizes a PAMP) and release inflammatory mediators responsible for the clinical signs of inflammation.

Chemical factors produced during inflammation (histamine, bradykinin, serotonin, leukotrienes, and prostaglandins) sensitize pain receptors, cause local vasodilation of the blood vessels, and attract phagocytes, especially neutrophils. Neutrophils then trigger other parts of the immune system by releasing factors that summon additional leukocytes and lymphocytes. Cytokines produced by macrophages and other cells of the innate immune system mediate the inflammatory response.

The complement system is a biochemical cascade of the immune system that helps, or "complements", the ability of antibodies to clear pathogens or mark them for destruction by other cells. The cascade is composed of many plasma proteins, synthesized in the liver, primarily by hepatocytes. The proteins work together to:

- trigger the recruitment of inflammatory cells
- "tag" pathogens for destruction by other cells by *opsonizing*, or coating, the surface of the pathogen
- form holes in the plasma membrane of the pathogen, resulting in cytolysis of the pathogen cell, causing the death of the pathogen

There are three different complement systems: Classical, alternative, Lectin

- Classical: starts when antibody bind to bacteria
- Alternative: starts "spontaneously"
- Lectin: starts when lectins bind to mannose on bacteria

Elements of the complement cascade can be found in many non-mammalian species including plants, birds, fish, and some species of invertebrates.

All white blood cells (WBCs) are known as leukocytes. Most leukocytes differ from other cells of the body in that they are not tightly associated with a particular organ or tissue; thus, their function is similar to that of independent, single-cell organisms. Most leukocytes are able to move freely and interact with and capture cellular debris, foreign particles, and invading microorganisms (although macrophages, mast cells, and dendritic cells are less mobile). Unlike many other cells in the body, most innate immune leukocytes cannot divide or reproduce on their own, but are the products of multipotent hematopoietic stem cells present in the bone marrow.

The innate leukocytes include: natural killer cells, mast cells, eosinophils, basophils; and the phagocytic cells include macrophages, neutrophils, and dendritic cells, and function within the immune system by identifying and eliminating pathogens that might cause infection.

ACQUIRED IMMUNITY:

The **adaptive immune system**, also known as the **acquired immune system** or, more rarely, as the **specific immune system**, is a subsystem of the overall immune system that is composed of highly specialized, systemic cells and processes that eliminate pathogens or prevent their growth. The acquired immune system is one of the two main immunity strategies found in vertebrates (the other being the innate immune system).

Acquired immunity creates immunological memory after an initial response to a specific pathogen, and leads to an enhanced response to subsequent encounters with that pathogen. This process of acquired immunity is the basis of vaccination. Like the innate system, the acquired system includes both humoral immunity components and cell-mediated immunity components.

Unlike the innate immune system, the acquired immune system is highly specific to a particular pathogen. Acquired immunity can also provide long-lasting protection; for example, someone who recovers from measles is now protected against measles for their lifetime. In other cases it does not provide lifetime protection; for example, chickenpox. The acquired system response destroys invading pathogens and any toxic molecules they produce. Sometimes the acquired system is unable to distinguish harmful from harmless foreign molecules; the effects of this may be hayfever, asthma or any other allergy. Antigens are any substances that elicit the acquired immune response (whether adaptive or maladaptive to the organism). The cells that carry out the acquired immune response are white blood cells known as lymphocytes. Two main broad classes —antibody responses and cell mediated immune response—are also carried by two different lymphocytes (B cells and T cells). In antibody responses, B cells are activated to secrete antibodies, which are proteins also known as immunoglobulins. Antibodies travel through the bloodstream and bind to the foreign antigen causing it to inactivate, which does not allow the antigen to bind to the host.

In acquired immunity, pathogen-specific receptors are "acquired" during the lifetime of the organism (whereas in innate immunity pathogen-specific receptors are already encoded in the germline). The acquired response is called "adaptive" because it prepares the body's immune

system for future challenges (though it can actually also be maladaptive when it results in autoimmunity).

Acquired immunity is triggered in vertebrates when a pathogen evades the innate immune system and (1) generates a threshold level of antigen and (2) generates "stranger" or "danger" signals activating dendritic cells.

The major functions of the acquired immune system include:

- Recognition of specific "non-self" antigens in the presence of "self", during the process of antigen presentation.
- Generation of responses that are tailored to maximally eliminate specific pathogens or pathogen-infected cells.
- Development of immunological memory, in which pathogens are "remembered" through memory B cells and memory T cells.

In humans, it takes 4-7 days for the adaptive immune system to mount a significant response.

CELLS OF ACQUIRED IMMUNE SYSTEM:

The cells of the acquired immune system are T and B lymphocytes; lymphocytes are a subset of leukocyte. B cells and T cells are the major types of lymphocytes. The human body has about 2 trillion lymphocytes, constituting 20–40% of white blood cells (WBCs); their total mass is about the same as the brain or liver. The peripheral blood contains 2% of circulating lymphocytes; the rest move within the tissues and lymphatic system.

B cells and T cells are derived from the same multipotent hematopoietic stem cells, and are morphologically indistinguishable from one another until after they are activated. B cells play a large role in the humoral immune response, whereas T cells are intimately involved in cell-mediated immune responses. In all vertebrates except Agnatha, B cells and T cells are produced by stem cells in the bone marrow.

T progenitors migrate from the bone marrow to the thymus where they are called thymocytes and where they develop into T cells. In humans, approximately 1–2% of the lymphocyte pool recirculates each hour to optimize the opportunities for antigen-specific lymphocytes to find their specific antigen within the secondary lymphoid tissues. In an adult animal, the peripheral lymphoid organs contain a mixture of B and T cells in at least three stages of differentiation:

- naive B and naive T cells (cells that have not matured), left the bone marrow or thymus, have entered the lymphatic system, but have yet to encounter their cognate antigen,
- effector cells that have been activated by their cognate antigen, and are actively involved in eliminating a pathogen.
- memory cells – the survivors of past infections.

Antigen presentation:

Acquired immunity relies on the capacity of immune cells to distinguish between the body's own cells and unwanted invaders. The host's cells express "self" antigens. These antigens are different from those on the surface of bacteria or on the surface of virus-infected host cells ("non-self" or

"foreign" antigens). The acquired immune response is triggered by recognizing foreign antigen in the cellular context of an activated dendritic cell.

- With the exception of non-nucleated cells (including erythrocytes), all cells are capable of presenting antigen through the function of major histocompatibility complex (MHC) molecules.[4] Some cells are specially equipped to present antigen, and to prime naive T cells. Dendritic cells, B-cells, and macrophages are equipped with special "co-stimulatory" ligands recognized by co-stimulatory receptors on T cells, and are termed professional antigen-presenting cells (APCs).
- Several T cells subgroups can be activated by professional APCs, and each type of T cell is specially equipped to deal with each unique toxin or microbial pathogen. The type of T cell activated, and the type of response generated, depends, in part, on the context in which the APC first encountered the antigen.
- Dendritic cells engulf exogenous pathogens, such as bacteria, parasites or toxins in the tissues and then migrate, via chemotactic signals, to the T cell-enriched lymph nodes. During migration, dendritic cells undergo a process of maturation in which they lose most of their ability to engulf other pathogens, and develop an ability to communicate with T-cells. The dendritic cell uses enzymes to chop the pathogen into smaller pieces, called antigens. In the lymph node, the dendritic cell displays these non-self antigens on its surface by coupling them to a receptor called the major histocompatibility complex, or MHC (also known in humans as human leukocyte antigen (HLA)). This MHC: antigen complex is recognized by T-cells passing through the lymph node. Exogenous antigens are usually displayed on MHC class II molecules, which activate CD4+T helper cells.
- Endogenous antigens are produced by intracellular bacteria and viruses replicating within a host cell. The host cell uses enzymes to digest virally associated proteins, and displays these pieces on its surface to T-cells by coupling them to MHC. Endogenous antigens are typically displayed on MHC class I molecules, and activate CD8+ cytotoxic T-cells. With the exception of non-nucleated cells (including erythrocytes), MHC class I is expressed by all host cells.

B- lymphocytes:

B Cells are the major cells involved in the creation of antibodies that circulate in blood plasma and lymph, known as humoral immunity. Antibodies (also known as immunoglobulin, Ig), are large Y-shaped proteins used by the immune system to identify and neutralize foreign objects. In mammals, there are five types of antibody: IgA, IgD, IgE, IgG, and IgM, differing in biological properties; each has evolved to handle different kinds of antigens. Upon activation, B cells produce antibodies, each of which recognize a unique antigen, and neutralizing specific pathogens.

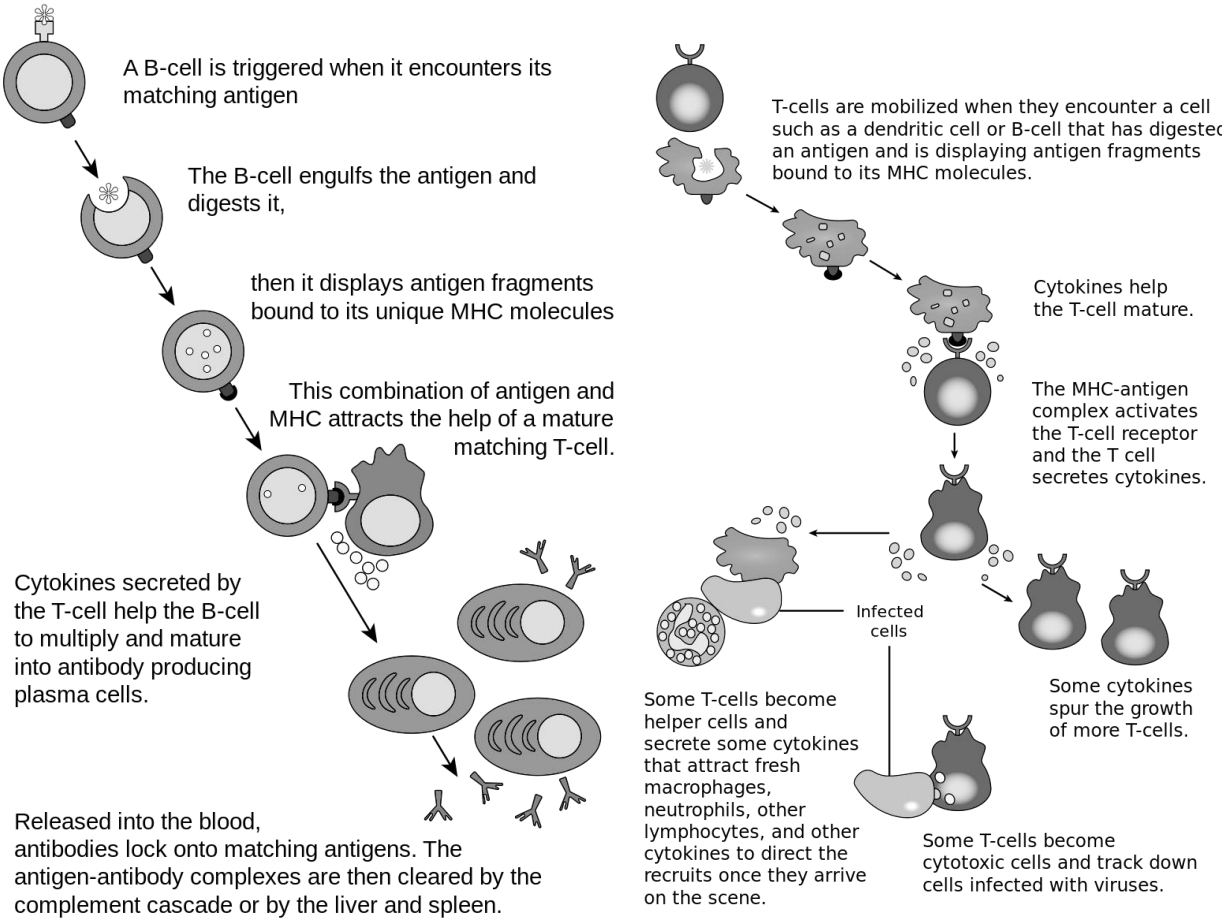
Antigen and antibody binding would cause five different protective mechanisms:

- Agglutination: Reduces number of infectious units to be dealt with
- Activation of complement: Cause inflammation and cell lysis
- Opsonization: Coating antigen with antibody enhances phagocytosis
- Antibody-dependent cell-mediated cytotoxicity: Antibodies attached to target cell cause destruction by macrophages, eosinophils, and NK cells

- Neutralization: Blocks adhesion of bacteria and viruses to mucosa

Like the T cell, B cells express a unique B cell receptor (BCR), in this case, a membrane-bound antibody molecule. All the BCR of any one clone of B cells recognizes and binds to only one particular antigen. A critical difference between B cells and T cells is how each cell "sees" an antigen. T cells recognize their cognate antigen in a processed form – as a peptide in the context of an MHC molecule, whereas B cells recognize antigens in their native form. Once a B cell encounters its cognate (or specific) antigen (and receives additional signals from a helper T cell (predominately Th2 type)), it further differentiates into an effector cell, known as a plasma cell.

Plasma cells are short-lived cells (2–3 days) that secrete antibodies. These antibodies bind to antigens, making them easier targets for phagocytes, and trigger the complement cascade. About 10% of plasma cells survive to become long-lived antigen-specific memory B cells. Already primed to produce specific antibodies, these cells can be called upon to respond quickly if the same pathogen re-infects the host, while the host experiences few, if any, symptoms.



B- Cell and T- Cell activation path ways

1.3. BASIC PROPERTIES OF ANTIGEN, STRUCTURE AND TYPES OF AN ANTIBODY

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.

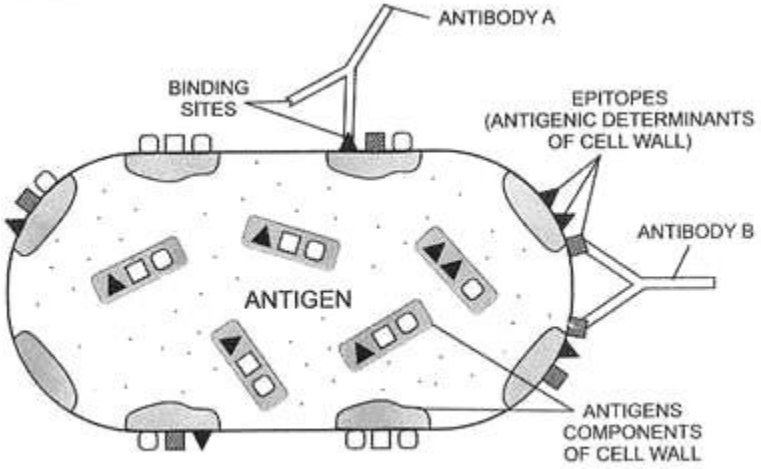


Diagram showing an antigen with epitopes (antigenic determinants). Two attached antibodies are also shown.\

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 I**mmunogens.**

Epitope is immunologically active regions of an immunogen (or antigen) that binds to antigen-specific membrane receptors on lymphocytes or to secreted antibodies. It is also called **antigenic determinants.**

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.

Adjuvants are substances that are non-immunogenic alone but enhance the immunogenicity of any added immunogen.

Chemical Nature of Antigens (Immunogens)

A. Proteins

The vast majority of immunogens are proteins. These may be pure proteins or they may be glycoproteins or lipoproteins. In general, proteins are usually very good immunogens.

B. Polysaccharides

Pure polysaccharides and lipopolysaccharides are good immunogens.

C. NucleicAcids

Nucleic acids are usually poorly immunogenic. However, they may become immunogenic when single stranded or when complexed with proteins.

D. Lipids

In general lipids are non-immunogenic, although they may be haptens.

Types of Antigen On the basis of order of their class (Origin)

1. Exogenous antigens
   - These antigens enters the body or system and start circulating in the body fluids and trapped by the APCs (Antigen processing cells such as macrophages, dendritic cells, etc.)
   - The uptakes of these exogenous antigens by APCs are mainly mediated by the phagocytosis
   - Examples: bacteria, viruses, fungi etc
   - Some antigens start out as exogenontigens, and later become endogenous (for example, intracellular viruses)
2. Endogenous antigens

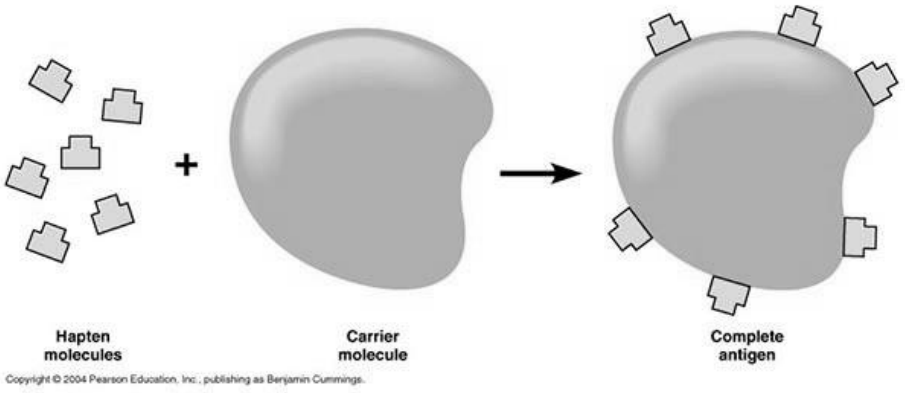
   - These are body's own cells or sub fragments or compounds or the antigenic products that are produced.
   - The endogenous antigens are processed by the macrophages which are later accepted by the cytotoxic T – cells.
   - Endogenous antigens include xenogenic (heterologous), autologous and idiotypic or allogenic (homologous) antigens.
   - Examples: Blood group antigens, HLA (Histocompatibility Leukocyte antigens), etc.
3. 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.

On the basis of immune response

1. Complete Antigen or Immunogen

   - Posses antigenic properties denovo, i.e. ther are able to generate an immune response by themselves.
   - High molecular weight (more than 10,000)
   - May be proteins or polysaccharides
2. Incomplete Antigen or Hapten



- These are the foreign substance, usually non-protein substances
- Unable to induce an immune response by itself, they require carrier molecule to act as a complete antigen.
- The carrier molecule is a non-antigenic component and helps in provoking the immune response. Example: Serum Protein such as Albumin or Globulin.
- Low Molecular Weight (Less than 10,000)
- Haptens can react specifically with its corresponding antibody.
- Examples: Capsular polysaccharide of pneumococcus, polysaccharide "C" of beta haemolytic streptococci, cardiolipin antigens, etc.

Determinants of Antigenicity

The whole antigen does not evoke immune response and only a small part of it induces B and T cell response.

The small area of chemical grouping on the antigen molecule that determines specific immune response and reacts specifically with antibody is called an ***antigenic determinant.***

Property of antigens/ Factors Influencing Immunogenicity

Immunogenicity is determined by:

1. Foreignness

- An antigen must be a foreign substances to the animal to elicit an immune response.

2. Molecular Size

- The most active immunogens tend to have a molecular mass of 14,000 to 6,00,000 Da.
- Examples: tetanus toxoid, egg albumin, thyroglobulin are highly antigenic.
- Insulin (5700) are either non-antigenic or weakly antigenic.

3. Chemical Nature and Composition

- In general, the more complex the substance is chemically the more immunogenic it will be.
- Antigens are mainly proteins and some are polysaccharides.

- It is presumed that presence of an aromatic radical is essential for rigidity and antigenicity of a substance.

4. Physical Form

- In general particulate antigens are more immunogenic than soluble ones.
- Denatured antigens are more immunogenic than the native form.

5. 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.

6. 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.

7. 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.

8. Auto-specificity

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

9. Genetic Factors

- Some substances are immunogenic in one species but not in another .Similarly, some substances are immunogenic in one individual but not in others (i.e. responders and non-responders).
- The species or individuals may lack or have altered genes that code for the receptors for antigen on B cells and T cells.
- They may not have the appropriate genes needed for the APC to present antigen to the helper T cells.

10. Age

- Age can also influence immunogenicity.
- Usually the very young and the very old have a diminished ability to elicit and immune response in response to an immunogen.

TYPES OF AN ANTIBODY:

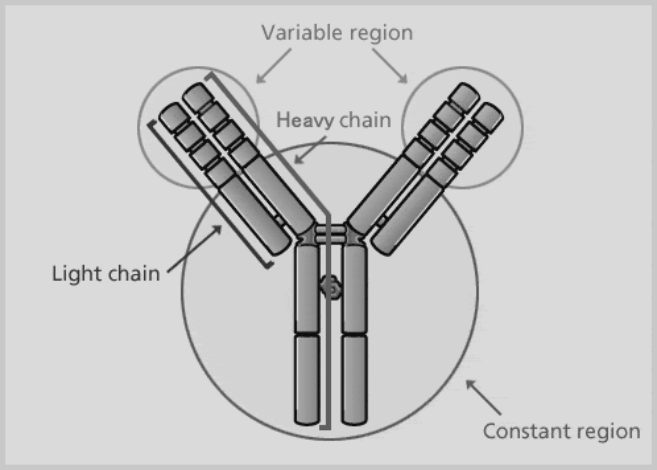
Antibody (Ab) also know as Immunoglobulin (Ig) is the large Y shaped protein produced by the body's immune system when it detects harmful substances, called antigens like bacteria and

viruses. The production of antibodies is a major function of the immune system and is carried out by a type of white blood cell called a B cell (B lymphocyte), differentiated B cells called plasma cells. The produced antibodies bind to specific antigens express in external factors and cancer cells.

Structure of Antibody

Antibodies are heavy (~150 kDa) globular plasma proteins. The basic structure of all antibodies are same.

There are four polypeptide chains: two identical **heavy chains** and two identical **light chains** connected by disulfide bonds. Light Chain (L) consists polypeptides of about 22,000 Da and Heavy Chain (H) consists larger polypeptides of around 50,000 Da or more. There are five types of Ig **heavy chain** (in mammal) denoted by the Greek letters: α, δ, ε, γ, and μ. There are two types of Ig **light chain** (in mammal), which are called lambda (andkappa(κ)) and kappa (κ).).
An antibody is made up of a variable region and a constant region, and the region that changes to various structures depending on differences in antigens is called the **variable region**, and the region that has a constant structure is called the **constant region.**



Each heavy and light chain in an immunoglobulin molecule contains an amino-terminal variable (V) region that consists of 100 to 110 amino acids and differ from one antibody to another. The remainder of each chain in the molecule – the constant (C) region exhibits limited variation that defines the two light chain subtypes and the five heavy chains subclasses. Some heavy chains (α, δ, γ) also contain a proline-rich hinge region. The amino terminal portions, corresponding to the V regions, bind to antigen; effector functions are mediated by the carboxy-terminal domains. The ε and μ heavy chains, which lack a hinge region, contain an additional domain in the middle of the molecule. CHO denotes a carbohydrate group linked to the heavy chain.

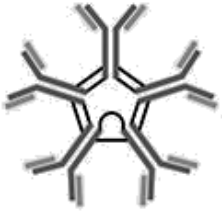
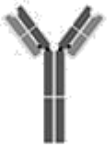
CLASSES/TYPES OF ANTIBODY

Serum containing antigen-specific antibodies is called antiserum. The 5 types – IgG, IgM, IgA, IgD, IgE – (isotypes) are classified according to the type of heavy chain constant region, and are distributed and function differently in the body.

Functions of Antibody

1. IgG provides long term protection because it persists for months and years after the prescence of the antigen that has triggered their production.
2. IgG protect against bacteris, viruses, neutralise bacterial toxins, trigger compliment protein systems and bind antigens to enhance the effectiveness of phagocytosis.
3. Main function of IgA is to bind antigens on microbes before they invade tissues. It aggregates the antigens and keeps them in the secretions so when the secretion is expelled, so is the antigen.
4. IgA are also first defense for mucosal surfaces such as the intestines, nose, and lungs.
5. IgM is involved in the ABO blood group antigens on the surface of RBCs.
6. IgM enhance ingestions of cells by phagocytosis.
7. IgE bind to mast cells and basophils wich participate in the immune response.
8. Some scientists think that IgE's purpose is to stop parasites.

The Five Immunoglobulin (Ig) Classes

	IgM pentamer	IgG monomer	Secretory IgA dimer	IgE monomer	IgD monomer
					
Heavy chains	μ	γ	α	ε	δ
Number of antigen binding sites	10	2	4	2	2
Molecular weight (Daltons)	900,000	150,000	385,000	200,000	180,000
Percentage of total antibody in serum	6%	80%	13%	0.002%	1%
Crosses placenta	no	yes	no	no	no
Fixes complement	yes	yes	no	no	no
Fc binds to		phagocytes		mast cells and basophils	
Function	Main antibody of primary responses, best at fixing complement; the monomer form of IgM serves as the B cell receptor	Main blood antibody of secondary responses, neutralizes toxins, opsonization	Secreted into mucus, tears, saliva, colostrum	Antibody of allergy and antiparasitic activity	B cell receptor

IgG

This isoform accounts for 70–75% of all human immunoglobulins found in the blood. Depending on the size of the hinge region, the position of disulfide bonds, and the molecular weight of the antibody, IgG can be further divided into 4 subclasses: IgG1, IgG2, IgG3, and IgG4.

In general, proteins are responsible for triggering IgG1 and IgG3 production, whereas IgG2 and IgG4 typically respond to foreign polysaccharides. IgG is the main component of the humoral immune system (immune response initiated by macromolecules present in the extracellular fluid) because of its abundance.

Due to its small size (monomeric) and high diffusibility, IgG is the prevalent type in the extracellular fluid that binds Fc receptors on phagocytic or other lytic cells and initiates the antibody-dependent cell-mediated cytotoxicity (ADCC) response – a cell-mediated defense mechanism wherein effector cells (phagocytes) destroy the target cell.

In addition, IgG triggers phagocytosis to initiate opsonization reaction – a process used to destroy foreign particles (e.g. bacteria) through phagocytosis. Apart from these functions, IgG is the only antibody that can cross the placenta and provides passive immunity to the fetus and infants in the first few months of life.

IgM

IgM is the largest antibody and the first one to be synthesized in response to an antigen or microbe, accounting for 5% of all immunoglobulins present in the blood. IgM typically exists as polymers of identical subunits, with a pentameric form as the prevalent one.

In its pentameric form, five basic antibody units are attached by disulfide bonds. Other forms include secretory IgM, which is synthesized by glandular-associated B cells, and monomeric form, which is present in the B cell membrane and functions as a B cell antigen receptor.

Due to its large size, IgM is mostly intravascular and has a lower affinity for antigens. However, since pentameric IgM has 10 antigen binding sites, it has higher avidity (overall binding strength) for antigens than IgG and acts as an excellent activator of the complement system and agglutination.

IgA

It accounts for 10–15% of all immunoglobulins and is prevalent in serum, nasal mucus, saliva, breast milk, and intestinal fluid. It has two subtypes namely IgA1 and IgA2, which mainly differ in terms of their hinge region characteristics. At mucosal surfaces, IgA provides the primary defense against inhaled and ingested pathogens.

IgE

IgE is the least prevalent one, with a serum concentration 10,000 times lower than IgG. However, the concentration of IgE increases significantly in allergic conditions, such as bronchopulmonary aspergillosis, and parasitic diseases, such as schistosomiasis.

In response to pathogens, IgE binds to mast cells via specific receptors, followed by pathogen-mediated cross-linking of these receptors (degranulation). This causes recruitment of eosinophil at the site of infection and destruction of pathogens via ADCC-type mechanisms.

IgD

IgD functions as a B cell antigen receptor and may participate in B cell maturation, maintenance, activation, and silencing. Although the exact function is still unclear, IgD may be involved in humoral immune responses by regulating B cell selection and homeostasis.

1.4. B AND T- CELL EPITOPS, HAPTENS, ADJUVANTS, ANTIGEN AND ANTIBODY REACTIONS

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. he epitopes of protein antigens are divided into two categories, conformational epitopes and linear epitopes, based on their structure and interaction with the paratope. Conformational and linear epitopes interact with the paratope based on the 3-D conformation adopted by the epitope, which is determined by the surface features of the involved epitope residues and the shape or tertiary structure of other segments of the antigen.

A conformational epitope is formed by the 3-D conformation adopted by the interaction of discontiguous amino acid residues. In contrast, a linear epitope is formed by the 3-D conformation adopted by the interaction of contiguous amino acid residues. A linear epitope is not determined solely by the primary structure of the involved amino acids. Residues that flank such amino acid residues, as well as more distant amino acid residues of the antigen affect the ability of the primary structure residues to adopt the epitope's 3-D conformation.

T cell epitopes are presented on the surface of an antigen-presenting cell, where they are bound to MHC molecules. In humans, professional antigen-presenting cells are specialized to present MHC class II peptides, whereas most nucleated somatic cells present MHC class I peptides. T cell epitopes presented by MHC class I molecules are typically peptides between 8 and 11 amino acids in length, whereas MHC class II molecules present longer peptides, 13-17 amino acids in length, and non-classical MHC molecules also present non-peptidic epitopes such as glycolipids.

HAPTEN :

Haptens are relatively small molecules that elicit an immune response only when attached to a large carrier such as a protein; the carrier may be one that also does not elicit an immune response by itself (in general, only large molecules, infectious agents, or insoluble foreign matter can elicit an immune response in the body). Once the body has generated antibodies to a hapten-carrier adduct, the small-molecule hapten may also be able to bind to the antibody, but it will usually not initiate an immune response; usually only the hapten-carrier adduct can do this. Sometimes the small-molecule hapten can even block immune response to the hapten-carrier adduct by preventing the adduct from binding to the antibody, a process called hapten inhibition.

The mechanisms of absence of immune response may vary and involve complex immunological mechanisms, but can include absent or insufficient co-stimulatory signals from antigen-presenting cells.

Haptens have been used to study allergic contact dermatitis (ACD) and the mechanisms of inflammatory bowel disease (IBD) to induce autoimmune-like responses.

The concept of haptens emerged from the work of Karl Landsteinerwho also pioneered the use of synthetic haptens to study immunochemical phenomena.

Examples of haptens:

A well-known example of a hapten is urushiol, which is the toxin found in poison ivy. When absorbed through the skin from a poison ivy plant, urushiol undergoes oxidation in the skin cells to generate the actual hapten, a reactive quinone-type molecule, which then reacts with skin proteins to form hapten adducts. Usually, the first exposure causes only sensitization, in which there is a proliferation of effector T-cells. After a subsequent, second exposure, the proliferated T-cells can become activated, generating an immune reaction that produces typical blisters of a poison ivy exposure.

Some haptens can induce autoimmune disease. An example is hydralazine, a blood pressure-lowering drug that occasionally can produce drug-induced lupus erythematosus in certain individuals. This also appears to be the mechanism by which the anaesthetic gas halothane can cause a life-threatening hepatitis, as well as the mechanism by which penicillin-class drugs cause autoimmune hemolytic anemia.

Other haptens that are commonly used in molecular biology applications include fluorescein, biotin, digoxigenin, and dinitrophenol.

Lastly, nickel allergy is caused by nickel metal ions penetrating the skin and binding to skin proteins.

ADJUVANTS:

An **adjuvant** is a pharmacological or immunological agent that modifies the effect of other agents. Adjuvants may be added to a vaccine to boost the immune response to produce more antibodies and longer-lasting immunity, thus minimizing the dose of antigen needed. Adjuvants may also be used to enhance the efficacy of a vaccine by helping to modify the immune response to particular types of immune system cells: for example, by activating T cells instead of antibody-secreting B cells depending on the purpose of the vaccine. Adjuvants are also used in the production of antibodies from immunized animals. There are different

classes of adjuvants that can push immune response in different directions, but the most commonly used adjuvants include aluminum hydroxide and paraffin oil.

Immunologic adjuvants are added to vaccines to stimulate the immune system's response to the target antigen, but do not provide immunity themselves. Adjuvants can act in various ways in presenting an antigen to the immune system. Adjuvants can act as a depot for the antigen, presenting the antigen over a longer period of time, thus maximizing the immune response before the body clears the antigen. Examples of depot type adjuvants are oil emulsions. An adjuvant can also act as an irritant, which engages and amplifies the body's immune response. A tetanus, diphtheria, and pertussis (DPT) vaccine, for example, contains small quantities of inactivated toxins produced by each of the target bacteria, but also contains some aluminium hydroxide.[4] Such aluminium salts are common adjuvants in vaccines sold in the United States and have been used in vaccines for more than 70 years.

ANTIGEN AND ANTIBODY INTERACTION:

Antigen-antibody interaction, or **antigen-antibody reaction**, is a specific chemical interaction between antibodies produced by B cells of the white blood cells and antigens during immune reaction. The antigens and antibodies combine by a process called agglutination.It is the fundamental reaction in the body by which the body is protected from complex foreign molecules, such as pathogens and their chemical toxins. In the blood, the antigens are specifically and with high affinity bound by antibodies to form an antigen-antibody complex. The immune complex is then transported to cellular systems where it can be destroyed or deactivated.

The first correct description of the antigen-antibody reaction was given by Richard J. Goldberg at the University of Wisconsin in 1952.It came to be known as "Goldberg's theory" (of antigen-antibody reaction).

There are several types of antibodies and antigens, and each antibody is capable of binding only to a specific antigen. The specificity of the binding is due to specific chemical constitution of each antibody. The antigenic determinant or epitope is recognized by the paratope of the antibody, situated at the variable region of the polypeptide chain. The variable region in turn has hyper-variable regions which are unique amino acid sequences in each antibody. Antigens are bound to antibodies through weak and noncovalent interactions such as electrostatic interactions, hydrogen bonds, Van der Waals forces, and hydrophobic interactions.

The principles of specificity and cross-reactivity of the antigen-antibody interaction are useful in clinical laboratory for diagnostic purposes. One basic application is determination of ABO blood group. It is also used as a molecular technique for infection with different pathogens, such as HIV, microbes, and helminth parasites.

1.5. B- CELL AND T- CELL ACTIVATION, MONOCLONAL ANTIBODIES AND THEIR PRODUCTION

B- CELL ACTIVATION:

B cell activation occurs in the secondary lymphoid organs (SLOs), such as the spleen and lymph nodes.[1] After B cells mature in the bone marrow, they migrate through the blood to SLOs, which receive a constant supply of antigen through circulating lymph. At the SLO, B cell activation begins when the B cell binds to an antigen via its BCR. Although the events taking place immediately after activation have yet to be completely determined, it is believed that B

cells are activated in accordance with the kinetic segregation model initially determined in T lymphocytes. This model denotes that before antigen stimulation, receptors diffuse through the membrane coming into contact with Lck and CD45 in equal frequency, rendering a net equilibrium of phosphorylation and non-phosphorylation. It is only when the cell comes in contact with an antigen presenting cell that the larger CD45 is displaced due to the close distance between the two membranes. This allows for net phosphorylation of the BCR and the initiation of the signal transduction pathway. Of the three B cell subsets, FO B cells preferentially undergo T cell-dependent activation while MZ B cells and B1 B cells preferentially undergo T cell-independent activation.

B cell activation is enhanced through the activity of CD21, a surface receptor in complex with surface proteins CD19 and CD81 (all three are collectively known as the B cell coreceptor complex).When a BCR binds an antigen tagged with a fragment of the C3 complement protein, CD21 binds the C3 fragment, co-ligates with the bound BCR, and signals are transduced through CD19 and CD81 to lower the activation threshold of the cell.

Once a BCR binds a TD antigen, the antigen is taken up into the B cell through receptor-mediated endocytosis, degraded, and presented to T cells as peptide pieces in complex with MHC-II molecules on the cell membrane. T helper (T_H) cells, typically follicular T helper (T_{FH}) cells recognize and bind these MHC-II-peptide complexes through their T cell receptor (TCR). Following TCR-MHC-II-peptide binding, T cells express the surface protein CD40L as well as cytokines such as IL-4 and IL-21. CD40L serves as a necessary co-stimulatory factor for B cell activation by binding the B cell surface receptor CD40, which promotes B cell proliferation, immunoglobulin class switching, and somatic hypermutation as well as sustains T cell growth and differentiation. T cell-derived cytokines bound by B cell cytokine receptors also promote B cell proliferation, immunoglobulin class switching, and somatic hypermutation as well as guide differentiation. After B cells receive these signals, they are considered activated. All these mechanism is considered as T- cell dependent activation of B-Cell.

T- CELL ACTIVATION:

Activation of $CD4^+$ T cells occurs through the simultaneous engagement of the T-cell receptor and a co-stimulatory molecule (like CD28, or ICOS) on the T cell by the major histocompatibility complex (MHCII) peptide and co-stimulatory molecules on the APC. Both are required for production of an effective immune response; in the absence of co-stimulation, T cell receptor signalling alone results in anergy. The signalling pathways downstream from co-stimulatory molecules usually engages the PI3K pathway generating PIP3 at the plasma membrane and recruiting PH domain containing signaling molecules like PDK1 that are essential for the activation of PKCθ, and eventual IL-2 production. Optimal $CD8^+$ T cell response relies on $CD4^+$ signalling.$CD4^+$ cells are useful in the initial antigenic activation of naïve CD8 T cells, and sustaining memory $CD8^+$ T cells in the aftermath of an acute infection. Therefore, activation of $CD4^+$ T cells can be beneficial to the action of $CD8^+$ T cells.

The first signal is provided by binding of the T cell receptor to its cognate peptide presented on MHCII on an APC. MHCII is restricted to so-called professional antigen-presenting cells, like dendritic cells, B cells, and macrophages, to name a few. The peptides presented to $CD8^+$ T cells by MHC class I molecules are 8–13 amino acids in length; the peptides presented to $CD4^+$ cells

by MHC class II molecules are longer, usually 12–25 amino acids in length,as the ends of the binding cleft of the MHC class II molecule are open.

The second signal comes from co-stimulation, in which surface receptors on the APC are induced by a relatively small number of stimuli, usually products of pathogens, but sometimes breakdown products of cells, such as necrotic-bodies or heat shock proteins. The only co-stimulatory receptor expressed constitutively by naïve T cells is CD28, so co-stimulation for these cells comes from the CD80 and CD86 proteins, which together constitute the B7 protein, (B7.1 and B7.2, respectively) on the APC. Other receptors are expressed upon activation of the T cell, such as OX40 and ICOS, but these largely depend upon CD28 for their expression. The second signal licenses the T cell to respond to an antigen. Without it, the T cell becomes anergic, and it becomes more difficult for it to activate in future. This mechanism prevents inappropriate responses to self, as self-peptides will not usually be presented with suitable co-stimulation. Once a T cell has been appropriately activated (i.e. has received signal one and signal two) it alters its cell surface expression of a variety of proteins. Markers of T cell activation include CD69, CD71 and CD25 (also a marker for Treg cells), and HLA-DR (a marker of human T cell activation). CTLA-4 expression is also up-regulated on activated T cells, which in turn outcompetes CD28 for binding to the B7 proteins. This is a checkpoint mechanism to prevent over activation of the T cell. Activated T cells also change their cell surface glycosylation profile. The T cell receptor exists as a complex of several proteins. The actual T cell receptor is composed of two separate peptide chains, which are produced from the independent T cell receptor alpha and beta (*TCRα* and *TCRβ*) genes. The other proteins in the complex are the CD3 proteins: CD3εγ and CD3εδ heterodimers and, most important, a CD3ζ homodimer, which has a total of six ITAM motifs. The ITAM motifs on the CD3ζ can be phosphorylated by Lck and in turn recruit ZAP-70. Lck and/or ZAP-70 can also phosphorylate the tyrosines on many other molecules, not least CD28, LAT and SLP-76, which allows the aggregation of signalling complexes around these proteins.

Phosphorylated LAT recruits SLP-76 to the membrane, where it can then bring in PLC-γ, VAV1, Itk and potentially PI3K. PLC-γ cleaves PI(4,5)P2 on the inner leaflet of the membrane to create the active intermediaries diacylglycerol (DAG), inositol-1,4,5-trisphosphate (IP3); PI3K also acts on PIP2, phosphorylating it to produce phosphatidlyinositol-3,4,5-trisphosphate (PIP3). DAG binds and activates some PKCs. Most important in T cells is PKCθ, critical for activating the transcription factors NF-κ).B and AP-1. IP3 is released from the membrane by PLC-γ and diffuses rapidly to activate calcium channel receptors on the ER, which induces the release of calcium into the cytosol. Low calcium in the endoplasmic reticulum causes STIM1 clustering on the ER membrane and leads to activation of cell membrane CRAC channels that allows additional calcium to flow into the cytosol from the extracellular space. This aggregated cytosolic calcium binds calmodulin, which can then activate calcineurin. Calcineurin, in turn, activates NFAT, which then translocates to the nucleus. NFAT is a transcription factor that activates the transcription of a pleiotropic set of genes, most notable, IL-2, a cytokine that promotes long-term proliferation of activated T cells.

PLCγ can also initiate the NF-κ).B pathway. DAG activates PKCθ, which then phosphorylates CARMA1, causing it to unfold and function as a scaffold. The cytosolic domains bind an adapter BCL10 via CARD (Caspase activation and recruitment domains) domains; that then binds TRAF6, which is ubiquitinated at K63. This form of ubiquitination does not lead to degradation of target proteins. Rather, it serves to recruit NEMO, IKKα and -β, and TAB1-2/

TAK1.TAK 1 phosphorylates IKK-β, which then phosphorylates Iκ).B allowing for K48 ubiquitination: leads to proteasomal degradation. Rel A and p50 can then enter the nucleus and bind the NF-κ).B response element. This coupled with NFAT signaling allows for complete activation of the IL-2 gene.

While in most cases activation is dependent on TCR recognition of antigen, alternative pathways for activation have been described. For example, cytotoxic T cells have been shown to become activated when targeted by other CD8 T cells leading to tolerization of the latter.

MONOCLONAL ANTIBODIES AND PRODUCTION:

Monoclonal antibodies (**mAb** or **moAb**) are antibodies that are made by identical immune cells that are all clones of a unique parent cell. Monoclonal antibodies can have monovalent affinity, in that they bind to the same epitope (the part of an antigen that is recognized by the antibody). In contrast, polyclonal antibodies bind to multiple epitopes and are usually made by several different plasma cell (antibody secreting immune cell) lineages. Bispecific monoclonal antibodies can also be engineered, by increasing the therapeutic targets of one single monoclonal antibody to two epitopes. It is possible to produce monoclonal antibodies that specifically bind to that substance; they can then serve to detect or purify that substance. This has become an important tool in biochemistry, molecular biology, and medicine. When used as medications, non-proprietary drug names.

Production of monoclonal antibodies involving human–mouse hybrid cells was first described by Jerrold Schwaber in 1973 and remains widely cited among those using human-derived hybridomas.

PRODUCTION:

Much of the work behind production of monoclonal antibodies is rooted in the production of hybridomas, which involves identifying antigen-specific plasma/plasmablast cells (ASPCs) that produce antibodies specific to an antigen of interest and fusing these cells with myeloma cells.Rabbit B-cells can be used to form a rabbit hybridoma. Polyethylene glycol is used to fuse adjacent plasma membranes,but the success rate is low, so a selective medium in which only fused cells can grow is used. This is possible because myeloma cells have lost the ability to synthesize **hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**, an enzyme necessary for the salvage synthesis of nucleic acids. The absence of HGPRT is not a problem for these cells unless the de novo purine synthesis pathway is also disrupted. Exposing cells to aminopterin (a folic acid analogue, which inhibits dihydrofolate reductase, DHFR), makes them unable to use the de novo pathway and become fully auxotrophic for nucleic acids, thus requiring supplementation to survive.

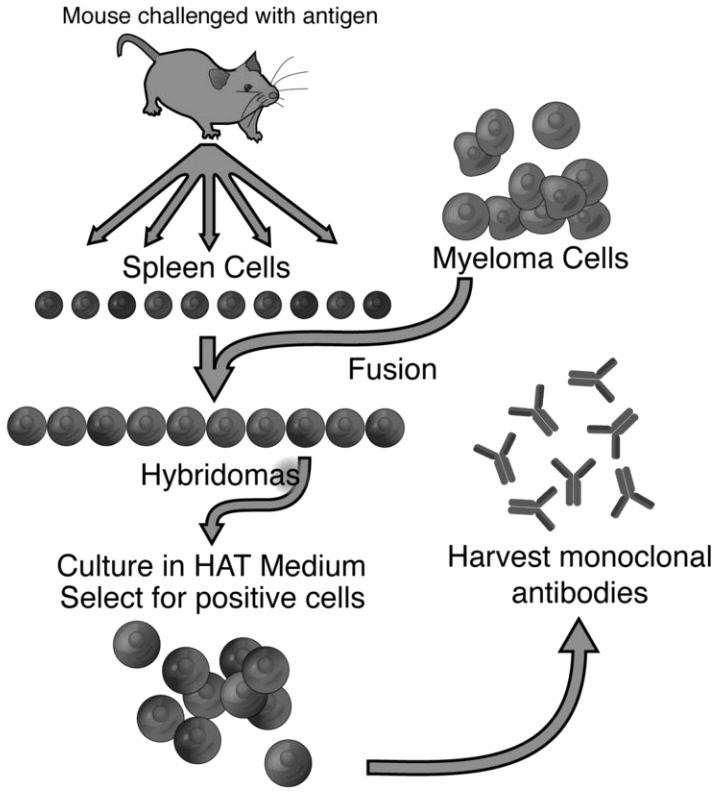
The selective culture medium is called HAT medium because it contains hypoxanthine, aminopterin and thymidine. This medium is selective for fused (hybridoma) cells. Unfused myeloma cells cannot grow because they lack HGPRT and thus cannot replicate their DNA. Unfused spleen cells cannot grow indefinitely because of their limited life span. Only fused hybrid cells, referred to as hybridomas, are able to grow indefinitely in the medium because the spleen cell partner supplies HGPRT and the myeloma partner has traits that make it immortal (similar to a cancer cell).

This mixture of cells is then diluted and clones are grown from single parent cells on microtitre wells. The antibodies secreted by the different clones are then assayed for their ability to bind to

the antigen (with a test such as ELISA or Antigen Microarray Assay) or immuno-dot blot. The most productive and stable clone is then selected for future use.

The hybridomas can be grown indefinitely in a suitable cell culture medium. They can also be injected into mice (in the peritoneal cavity, surrounding the gut). There, they produce tumors secreting an antibody-rich fluid called ascites fluid.

The medium must be enriched during *in vitro* selection to further favour hybridoma growth. This can be achieved by the use of a layer of feeder fibrocyte cells or supplement medium such as briclone. Culture-media conditioned by macrophages can be used. Production in cell culture is usually preferred as the ascites technique is painful to the animal. Where alternate techniques exist, ascites is considered unethical.



A representation of monoclonal antibody production

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CORE PAPER-VII

IMMUNOLOGY AND ANIMAL BIOTECHNOLOGY(UNIT-II)

MAJOR HISTOCOMPATIBILITY COMPLEX

Whenever a pathogen is taken by the cell such as Macrophages. It gets broken down by the action of enzymes released from Lysosomes. These broken pieces are called "Antigenic determinants" (Epitopes).

This is called processing of Antigens. These antigenic determinants are called "MHC " molecules, Majorhisto-compatibility complex .

It is discovered by "Gorer" in 1936 in inbred mice strains. without MHC molecules there would be no presentation of internal or external antigens to the T-cells.MHC proteins allow T-cells to distinguish self from non-self.

STRUCTURE:MHC is Polymorphic. It contains several different MHC class-I and MHC class-II genes ,so that every individual possess a set of MHC molecules with different ranges of peptide-binding specificities.

MHC is highly polymorphic. These are multiple variants of each gene ,with in the population as a *whole.MHC genes are in fact the most polymorphic genes known.*

The MHC is located on chromosome-6 in Humans and chromosome-17 in the mouse. In Humans it contains more than 200 genes.

Recent studies suggest that MHC may span at least $7x10^6$ base pairs. The genes encoding α chains of MHC class-I and β chains of MHC class-II molecules are linked with in the complex, the gene for β-micro-globulin and invariant chain are on different chromosomes-15 and 5.

These genes are called Human Leucocyte Antigen or HLA genes as first discovered through antigenic differences between White blood cells from different individuals.

There are 3calss-Iα-Chain genes in humans called HLA-A,-B,-C. These are responsible for the production of *Ag****'s on surface*** ***<u>of nucleated cells</u>*** *and* ***causing transplantation reactions hence called transplantation antigens.***

There are also 3 pairs of MHC class-II α and β-chain genes called HLA-DR,DP and DQ. These produce antigens called Immune associated antigen(Ia).These antigens are associated with the regulation of Immune response. These antigens are present on surface of B-cells, Macrophages , Monocytes , Antigen presenting cells and activated T-cells.

MHC class III genes are not related structurally to class-I and class-II and have no role in Antigen presentation ,although most play same role in immune responses.C_2, C_{4a}, C_{4b}, factor-B , 21-hydroxylase enzymes.TNF α, TNF β heat shock proteins(HSP).

TLa *class-IV genes located adjacent to A is associated with antigens present on T-cells of Leukemia (TLa) as well as on immature thymocytes which lose TLa during differentiation into immature cells.*

TYPES OF MHC

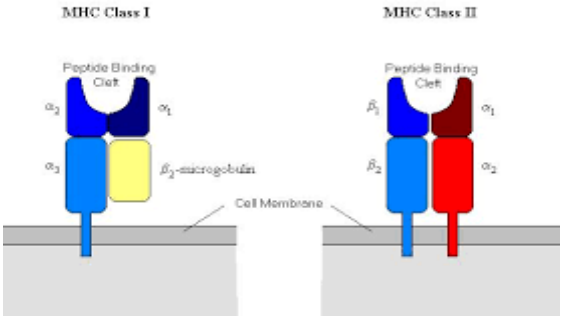
The immune system is able to identify non-self cells by aberrations in MHC displayed on plasma membrane. There are 2 groups of major MHC molecules , each group generates different markings on the plasma membrane. They are:

1)MHC class-I molecules: These are membrane bound glycoprotein in nature and are found on cell surface of all nucleated cells and are one of the main 2 classes of MHC. They don't occur in RBC's, but present in platelets .These are composed of 2 non-identical chains, a long α chain and a short β chain encoded by HLA(HUMAN

LEUCOCYTE)antigen genes HLA-A, HLA-B,HLA-C. The α chain is coded on locus of MHC in chromosome-6 and β chain encoded on chromosome-15.

2)MHC class-II molecules: These are expressed by specialized type of cells known as Antigen presenting cells such as Macrophages ,B-cells and dendritic cells.

These molecules possess 2 identical α and β chains encoded by MHC locus of chromosom-6.These are encoded by gene HLA-D.



FUNCTIONS OF MHC MOLECULES

Functions of class-I MHC molecules: These present the antigens to T_C cells for cell mediated immunity. the following are also the functions of these molecules.

1)Initiation of immunological functions :These molecules detect protein fragments from non-self proteins with in the cell. These fragments are known as Antigens. Non-self proteins detected by these molecules are situated on T_C cells. T_c cells possess co-receptor molecules CD_8.MHCclass-I molecules present Antigens onCD_8 receptors that will initiate immunological reactions .

2)Production of Antibodies: These molecules identify almost all the cells of the body as self. These induce production of antibodies which are introduced into the host with different class-I molecules.

3)Peptide binding: These function as messenger in displaying intra cellular proteins to T_c cells to prevent immunological responses when intra-cellular proteins degrade by proteosome ,peptide particles bind to MHC-I. Such particles are the Epitopes.

4)T-cell tolerance: MHC-I protein complex is presented into the external plasma membrane of cell through endoplasmic reticulum. Then epitope bind t on extra cellular surfaces of MHC-I molecules.T_C cells are not activated in response to self-antigens i.e. T_C cell tolerance.

5)Auto-immunity: These partly determine the response of an individual to antigens of infectious microorganisms. Therefore ,it is implicated in susceptibility to disease and in development of Auto-immunity.

FUNCTIONS OF CLASS-II MHC MOLECULES

MHC class-II molecules functions: these are found on all APC'S in addition to MHC-I molecules. They present antigens to T_H cells for cell mediated and humoral immunity. They are helpful in----------

1)Creating immunological responses: MHC-I molecules present antigen to other cells of immune system to initiate immunological response with the help of $T_H$cells.

2)clear exogenous antigens :These clear exogenous antigens to CD_4Tcells.

3)Apoptosis: These molecules process endognous antigens to CD_8T cells in order to make infected cell undergo apoptosis by inducing release of perforins and granzymes.

4) production of Antibodies: These comprise of D group of MHC-II .These stimulate the production of antibodies but they are required for T-cell communication with Macrophage and B-cells.

FUCTIONS OF CLASS-III MHC MOLECULES:

These partiiipate(C_2. C_{4a}, C_{4b})in the ilassiial pathway and faitor-B in alternate pathway of immune responses.

BASIC PROPERTIES AND FUNCTIONS OF CYTOKINES

Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They must be produced de novo in response to an immune stimulus. They generally (although not always) act over short distances and short time spans and at very low concentration. They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules.

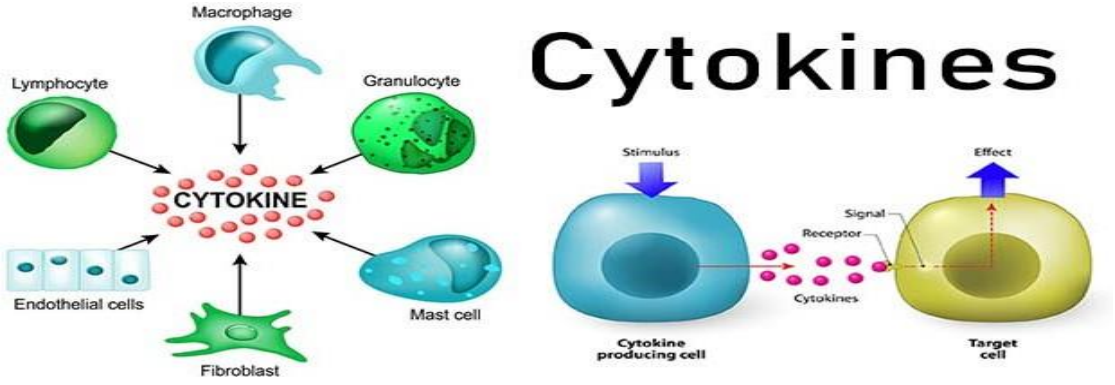
Cytokines are named as-

Lymphokine (cytokines made by lymphocytes),

Monokine (cytokines made by monocytes),

Chemokine (cytokines with chemotactic activities),

Interleukin (cytokines made by one leukocyte and acting on other leukocytes).



PROPERTIES OF CYTOKINES:

1) Target specific and induce signal transduction: Cytokines binds to pacific receptor on the cell membrane of target cell which triggers signal transduction pathway that ultimately alter gene expression in target cell.

2) High affinity: The affinity between cytokines add their receptors is very high. Because of high affinity, cytokines can mediate biological effects at Pico molar concentration.

3) Action: A particular cytokine possess the following action.

i)Autocrine action: Cytokines may act on the cells that secrete them.

ii) Paracrine action: cytokines may bind to the receptor on target cell in close proximity to produce.

iii) Endocrine action: cytokines act in some instances on distant cells .

4) Attributes :cytokines exhibit induction of following attributes.

i) pleiotropy: It is common for different cell types to secrete the same cytokine or for a single cytokine to act on several different cell types .

ii) Redundancy: Cytokines are redundant in their activity, meaning similar functions can be stimulated by different cytokines.

iii) Cascade: Cytokines are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines.

IV) SYNERGY:

Cytokines can also act synergistically (two or more cytokines acting together)

V)Antagonism: The effect of one cytokine inhibit the effect of other.

FUNCTIONS OF CYTOKINES

Biological functions include

- ***Major role in induction and regulation of cellular interactions involving cells of immune, inflammatory and haemopioetic systems.***
- ***Exhibit pleiotrophy, redundancy, synergy, antagonism.***
- ***Healing of wounds.***
- ***Control cellular proliferation and differentiation.***

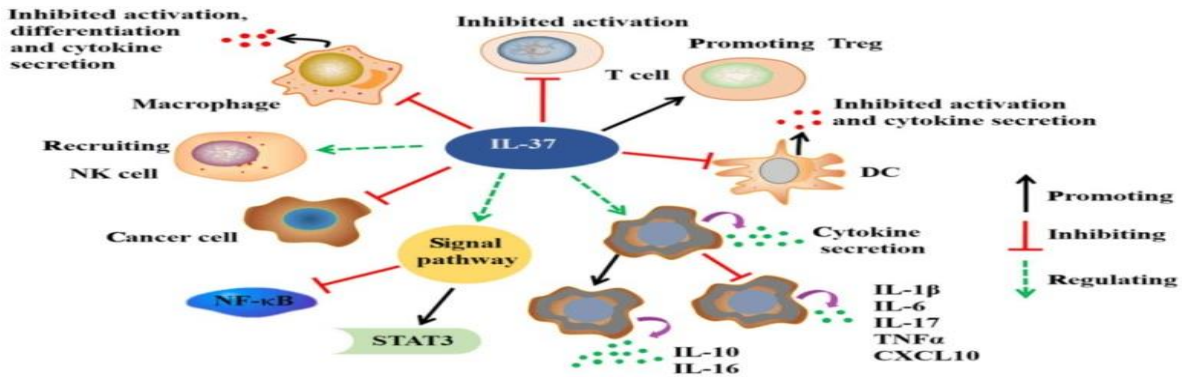
PRINCIPAL BIOLOGICAL ACTIONS

1) Cytokines acting as mediators and regulators of innate immunity:
Different cytokines that regulate and mediate innate immunity are produced by mononuclear phagocytes in response to microbial agents such as lipo-polysaccharide of bacteria ,ds-RNA of virus etc. These act on endothelial cells ,leucocytes to stimulate early inflammatory reactions to microbes, and some functions to control these responses.

2) Act as mediators and regulators of Adaptive immunity: These are mainly produced by T-lymphocytes in response to specific recognition of foreign antigens. Many of such cytokines regulate growth, differentiation of various lymphocyte. Thus stimulate activation phase of T-cell dependent immune responses .Other cytokines may activate and regulate specialized effector cells like mono-nuclear phagocytes, neutrophils and eosinophils to eliminate antigens in effector phase of adaptive immunity.

3) Act as stimulators of Haematopoiesis: Haemato-poeisis stimulating cytokines are produced by bone marrow stromal cells, leucocytes and other cells. These cause stimulation of growth, differentiation of immature leucocytes.

The cytokines of innate and adaptive immunity are produced by different cell population and act on different target cells.



NOTE:Cytokines that initiate activation or differentiation of cells of immune system to stimulate phagocytosis and cytolysis of infected cells are mainly of two types i)Interleukins ii) Interferons.

Interleukins' (ILs)

Any of a group of naturally occurring proteins that mediate communication between cells. Interleukins regulate cell growth, differentiation, and motility. They are particularly important in stimulating immune responses, such as inflammation. Interleukins are a subset of a larger group of cellular messenger molecules called cytokines, which are modulators of cellular behavior. Fifteen different types of interleukins are known, and they are designated numerically, IL-1 through IL-15. The immunological functions of most of the interleukins are known to some degree. IL-1 and IL-2 are primarily responsible for activating T and B lymphocytes (white blood cells integral to bringing about the acquired immune response), with IL-2 being a stimulant of T- and B-cell growth and maturation. IL-1, along with IL-6, is also a mediator of inflammation. IL-4 often leads to an increase in antibody secretion by B lymphocytes, while IL-12 causes a greater number of the leukocytes cytotoxic T cells and natural killer cells to be made. The set of interleukins stimulated by a specifc infectious agent determines which cells will respond to the infection and influences some of the clinical manifestations of the disease.

WHAT ARE INTERFERONS?

Interferons (IFN's)are a group of signaling made and released by host cells in response to the presence of several viruses .In a typical scenario, a virus-infected cell will release interferons causing nearby cells to heighten their anti-viral defenses.

IFNs belong to the large class of proteins known as cytokines . molecules used for communication between cells to trigger the protective defenses of the immune system that help eradicate pathogens. Interferons are named for their ability to "interfere" with viral replication by protecting cells from virus infections.

IFNs also have various other functions like:

They activate immune cells such as natural killer and macrophages; they increase host defenses by up-regulating antigen presentation by virtue of increasing the expression of major histo- compatibility complex (MHC) antigens. Certain symptoms of infections, such as fever, muscle pain, and "flu-like symptoms", are also caused by the production of IFNs and other cytokines.

More than twenty distinct IFN genes and proteins have been identified in animals, including humans. They are typically divided among three classes: Type I IFN, Type II IFN, and Type III IFN. IFNs belonging to all three classes are important for fighting viral infection and for the regulation of the immune system

TYPES OF INTERFERONS

Based on the type of receptor through which they signal, human interferon's have been classified into three major types.

- *Interferon type I: All type- I IIFNs bind to a specific cell surface receptor complex known as the IFN-α/β receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains.*
- *The type -I interferons present in humans are IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω.*
- *In general, type I interferons are produced when the body recognizes a virus that has invaded it. They are produced by fibroblasts and monocytes. However, the production of type I IFN-α is prohibited by another cytokine known as Interleukin-10. Once released, type- I interferons bind to specific receptors on target cells, which leads to expression of proteins that will prevent the virus from producing and replicating its RNA and DNA. Overall, IFN-α can be used to treat hepatitis B and C infections, while IFN-β can be used to treat multiple sclerosis.*
- *Interferon type- II (IFN-γ in humans): This is also known as immune interferon and is activated by Interleukin-12. Furthermore, type II interferons are released by Cytotoxic T cells and T helper cells, type 1 specifically. However, they block the proliferation of T helper cells type two. The previous results in an inhibition of T_h2 immune response and a further induction of T_h1 immune response, which leads to the development of debilitating diseases such as multiple sclerosis. IFN type II binds to IFNGR, which consists of IFNGR1 and IFNGR2 chains.*
- *Interferon type- III: Signal through a receptor complex consisting of IL10R2 (also called CRF2-4) and IFNLR1 (also called CRF2-12). Although discovered more recently than type I and type II IFNs , recent information demonstrates the importance of Type III IFNs in some types of virus or fungal infections.*

In general, type I and II interferons are responsible for regulating and activating the immune response.

Expression of type I and III IFNs can be induced in virtually all cell types upon recognition of viral components, especially nucleic acids, by cytoplasmic and endosomal receptors, whereas type II interferon is induced by cytokines such as IL-12, and its expression is restricted to immune cells such as T cells and NK cells.

FUNCTIONS OF INTERFERONS

- ***Interferons have antiviral properties and anti-proliferative effects on cell division.***
- ***They can activate T-cell ,neutrophils, NK cells***
- ***They can activate and enhance phagocytic properties of Macrophages.***
- ***Increase antigen-presenting capacity of APC'S.***
- ***Increase expression of MHC class-I and II molecules in various cells.***

Clinically useful in:

- ***Treatment of Hepatitis-B and C-IFN-α***
- ***Treatment of multiple sclerosis and autoimmune disorders-IFN-β***
- ***Have anti tumor properties.***
- ***Treatment of cancers ,in combination with chemotherapy.***

COMPLEMENT PROTEINS

These are a group of inactive plasma proteins, cell surface proteins and are large thermo labile enzymatic proteins .

Discovered by Bordet in 1895 and named it as Alexin. Term complement was coined by Ehrlich.

These when activated form a membrane attack complex(MAC)that form pores in the plasma membrane of affected cells allowing ECF to enter the cells and make them well and burst, Some of them form a coat on surface of pathogens and attract neutrophils and macrophages to phago-cytose and destroy them(OPSONIZATION).Complement proteins and their activities are together called Complement system.

SALIENT FEATURES OF COMPLEMENT PROTEINS

These are characterized by following features:

1. ***Found in normal serum***
2. ***They are β-globulins***

3. *Neither antigens nor antibodies.*
4. *Exist as pro-enzymes and circulate as in active forms*
5. *On activation ,acquire enzymatic/esterase activity*
6. *Activated components are shown by putting a bar over them*

Eg: $\bar{C}_3, \bar{C}_2$ *etc*

7. *Inactive forms are indicated by suffix –i*

Eg: C_{4i}, C_{3i}

8. *These are thermo-labile and destroyed at room temperature or at 56°c.*
9. *complement system is formed by 20 different types of (β-globulins) proteins.*
10. *out of these 16 are biologically important and are designated by letter 'C'.*

Numbered C_1 ---------- C_9

11. C_1 *consists of 3 sub-units, namely* C_{1q}, C_{1r} *and* C_{1s}.
12. *These proteins are capable of reacting each –other in a particular sequence as C* C_{1q}, C_{1r}, C_{1s}, C_4, C_2, C_3, C_5, C_6, C_7, C_8, C_9
13. *Other components include B,D,P,H,I.*
14. *During sequential activation ,a number of biological events are initiated which facilitate the destruction of foreign materials by the cells of immune system.*
15. *These have no micro -bicidal effect but help in action of various types of antibodies.*

FUNCTIONS OF COMPLMENT SYSTEM

Some major functions of complements are:

1. *Opsonization and phagocytosis: C3b, bound to immune complex or coated on the surface of pathogen, activate phagocytic cells. These proteins bind to specific receptors on the phagocytic cells to get engulfed.*
2. *Cell lysis: Membrane attack complex formed by C5b6789 components ruptures the microbial cell surface which kills the cell.*
3. *Chemo taxis: Complement fragments attract neutrophils and macrophages to the area where the antigen is present. These cell surfaces have receptors for complements, like C5a, C3a, thus, run towards the site of infammation, i.e. chemo taxis.*
4. *Activation of mast cells and basophils and enhancement of infammation: The proteolytic complement fragments, C5a, C4a, and C3a induce acute infammation by activating mast cells and neutrophils. All three peptides bind to mast cells and induce degranulation, with the release of vasoactive mediators such as histamine. These peptides are also called anaphylatoxins because the mast cell reactions they trigger are characteristic of anaphylaxis. Binding to specific complement receptors on cells of the immune system, they trigger specific cell functions, infammation, and secretion of immunoregulatory molecules.*
5. *Production of antibodies: B cells have receptor for C3b. When C3b binds to B-cell, it secretes more antibodies. Thus C3b is also an antibody producing amplifiers which converts it into an effective defense mechanism to destroy invading microorganism.*
6. *Immune clearance: The complement system removes immune complexes from the circulation and deposits them in the spleen and liver. Thus it acts as anti-infammatory function. Complement proteins promote the solubilization of these complexes and their clearance by phagocytes.*
7. *Aaphylactoid reaction :* C_{3a}, C_{5a} *cause release of of histamine from mast cells. They have kinin like activity and chaemotactic activity .cause edema, smooth muscle dilation ,infammation.*

8. *Coagulation: blood platelets adhered to activated* C_3 *are lysed and release factor that convert prothrombin to thrombin that bring about coagulation.*

Complement regulation

The complement system has the potential to be extremely damaging to host tissues; hence regulatory mechanisms are required to restrict the complement pathway. Various plasma and cell membrane proteins regulate complement activation by inhibiting different steps in the cascade.

The membrane of most mammalian cells has a high level of sialic acid, which contributes to the inactivation of complements.

Complement related Diseases

Diseases associated with complements can be due to the deficiencies in any of the protein components or in regulatory components.

Some examples of complement protein deficiencies are:

Deficiency of C2 and C4 can cause systemic lupus erythematosus; deficiency of C3 and factor D can cause pyogenic bacterial infection; and deficiency of C5-C9 (or MAC deficiency) may lead to the Neisserial infections like, gonorrhea and meningitis.

Deficiencies of regulatory proteins lead to too much activation of complements in wrong time and place which leads to unwanted inflammation and cell lysis. Pyogenic bacterial infection and glomerulonephritis are the results of such deficiencies.

Mutations in the complement regulators factors may lead to atypical hemolytic uremic syndrome, age-related macular degeneration, hereditary angio-edema, etc.

Complement system can also be stimulated by abnormal stimuli, like persistent microbes, antibody against self antigens or immune complexes deposited in tissues. Even when the system is properly regulated and activated, it can cause significant tissue damage.

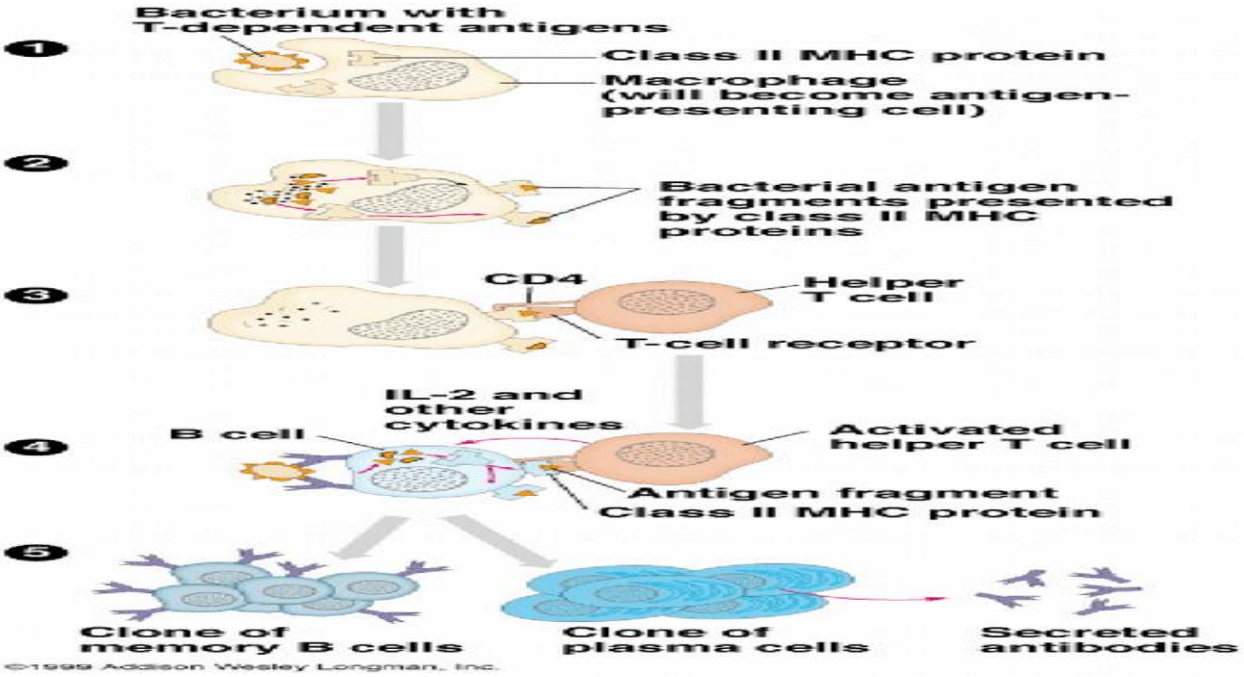
TYPES OF IMMUNE RESPONSES

Immune responses can be categorized into 2 types i)specific and ii)non-specific

Specific immune responses are again 2 types 1) Humoral2)cell-mediated

1) HUMORAL IMMUNITY (Antibody-mediated immunity) :In the secondary lymphoid organs, the free circulating antigens bind to F_{ab} end of the antibodies that are present on surface of mature B-cells. They engulf and process antigens. Then they display on their membrane with the antigenic fragments on their membrane with the help of class MHC class-II molecules.T_H cells recognize them interact with the antigen-MHC-II complex and release a type of interleukin, which stimulate B-cells to proliferate and differentiate into Memory cells and Plasma cells. Plasma cells release specific antibodies into the plasma or extra cellular fluids. These antibodies help in opsonization, immobilization of bacteria, neutralizing, cross-linking of antigens leading to agglutination of insoluble antigens and precipitation of soluble antigens. These also activate phagocytes and complement system.

Note :Antigen –antibody complexes /immunocomplexes are removed by eosinoplhils and monocytes.



ANTIGEN-ANTIBODY REACTIONS :

***Neutralization:** It refers to the ability of specific antibodies, called Neutralizing antibody, to bind the antigen and neutralize its biological effects by binding the epitope. However, these antibodies are not killing or destroying the antigen. The binding antibodies, on the counterpart, binds the antigen and attracts the other immune cells to kill the cells.*

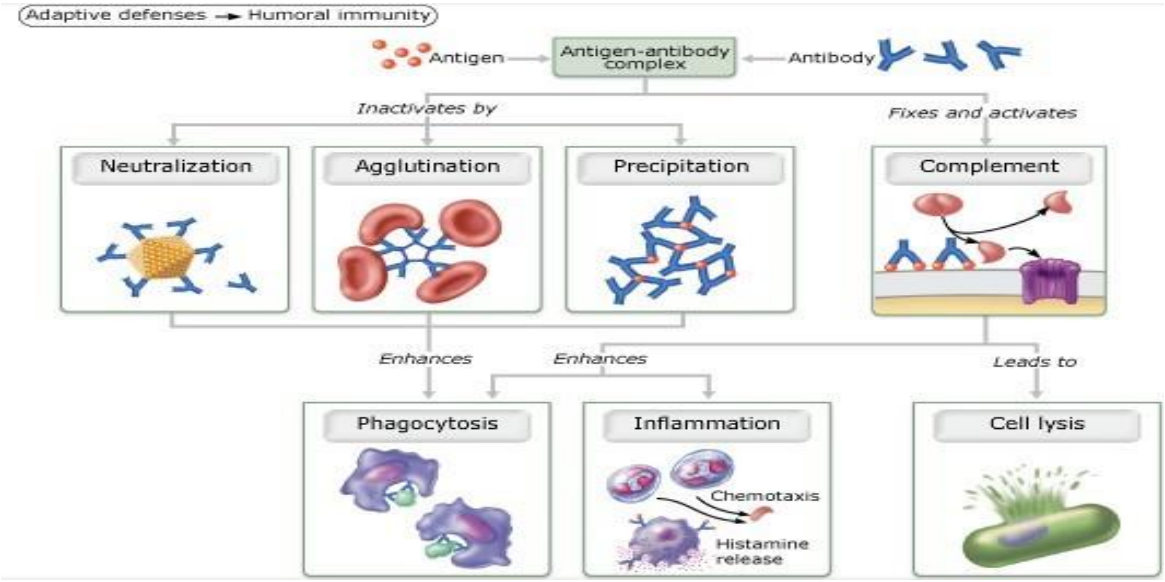
"Antibodies can simply bind to IFN-beta or glatiramer acetate (binding Ab, or BAb) with no subsequent effect on function, or they can block or neutralize (neutralizing Ab, or NAb) their biological activity."

***Agglutination:** In Agglutination, when any antibody binds the antigen, they will facilitate the aggregation of the other antigen-antibody complex such that all the antigen are clubbed together. It makes easy for the immune system to clear the antigen from the site of infection.*

***Opsonization:** In this case, the antigen or the pathogen will be marked with a certain class of proteins such as Opsonin which would stimulate the process of Phagocytosis by the special type of immune cells called Phagocytes. Every microbe has certain specific molecules called Pathogen-associated Molecular Patterns (PAMPs). These PAMPs are identified by the Pattern Recognition Receptors (PRR) of Phagocytes. One novel example of PAMPs is bacterial LPS (Lipopolysaccharide) called endotoxin, and they are recognized by the TLR4 (Toll like receptor-4) proteins.*

Other PAMPs include bacterial fagellin (recognized by TLR5), lipoteichoic acid from gram-positive bacteria, peptidoglycan, and nucleic acid variants normally associated with viruses, such as double-stranded RNA (dsRNA), recognized by TLR3 or unmethylated CpG motifs, recognized by TLR9

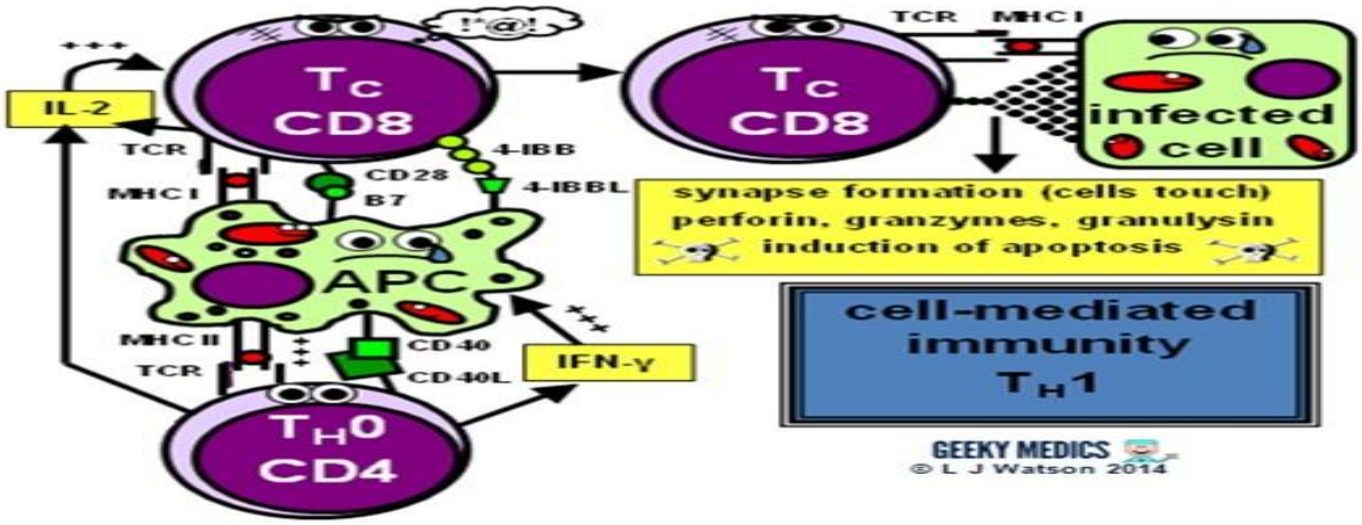
***Precipitation:** Binding of antibody with antigen form antigen-antibody complex (precipitins) which is heavier and precipitate so that phagocytic cells ingest it more readily.*



CELL MEDIATED IMMUNITY

Immunity mediated by the activated T-cells, natural killer cells, etc., is known a cell mediated immunity-effective against both exogenous with the help of MHC-II Endogenous antigens with the help of class MHC-I. It does not involve in production of antibodies.

Mechanism: Antigen presenting cells process exogenous antigens where as the altered self cells process endogenous antigens .T hen the processed antigenic fragments are displayed on their (APC's or AC's)membranes. They are recognized by T-cells. Binding of T-cells to the APC's or ASC' s causes the production of activated T cells and memory T cells. The activated T_H cells secrete various types of interleukins which transform activated T_c cells into effector Cyto -toxic T lymphocytes(CTLs/Killer cells).CTLs attach to the infected cells and release certain enzymes called Perforins and Granzymes. Perforins form pores in the cell membrane of the infected cells. Then granzymes enter enter infected cells through these perforations and activate certain proteins(caspases) which help in destruction of infected cell(Apoptosis).The NK cells are similar in their action to CTLs.



Biological functions of cell-mediated immunity:

1) Immune survivellance: Careful watching, recognition, and killing of tumor cells by immune system I called Immune survivelnce.Tumour antigen can stimulate cytotoxic T-cell and helper T-cell to kill tumor cells.

2) Graft rejections: The transplantation of tissues between genetically non-identical individuals of same species is called Allograft. Allograft decays and degenerates. It is called Graft rejection. It is bought about by Cell-mediated immune response. Allograft is rejected in 2 ways i) HLA antigen of allograft that stimulate T_H cell.T_H cell stimulate T_C that kill graft cells.

ii) Activated T_H cells also release Lymphokines that inturn stimulate Macrophages and makes them swallow the graft cells by phagocytosis.

3) Delayed hypersensitivity reaction: Delayed hypersensitivity reactions are inflammatory reactions initiated by mononuclear leukocytes. The term delayed is used to differentiate a secondary cellular response, which appears 48-72 hours after antigen exposure, from an immediate hypersensitivity response, which generally appears within 12 minutes of an antigen challenge. These reactions are mediated by T cells and monocytes/macrophages rather than by antibodies. They are also termed type IV hypersensitivity reactions. Delayed hypersensitivity is a major mechanism of defense against various intracellular pathogens, including mycobacteria, fungi, and certain parasites, and it occurs in transplant rejection and tumor immunity. The central role of CD4+ T cells in delayed hypersensitivity is illustrated in patients with AIDS.

HYPERSENSITIVITY

Our immune system works continuously to keep us healthy and protect us against bacteria, viruses, and other germs. Sometimes, however, this system becomes too sensitive, causing *hypersensitivity reactions* ***that can be harmful or even deadly. These reactions are the result of exposure to some type of foreign antigen either on or in the body.***

10./. human population suffer from hypersensitivity. clinically hypersensitivity is known as 'ALLERGY'.

Factors causing Hypersensitivity: Factors causing hypersensitivity are known as Allergens. These may be extrinsic or intrinsic.

- ***Drugs such as penicillin, sulphanamide, aspirin etc.***
- ***Airborne particles such as pollens, house dust, mites, spores, animal dander like fur, (scales, feathers.***
- ***Infectious organisms like bacterium, virus, fungi, parasites.***
- ***Food stuffs like strawberries, shellfish, brinjal etc.***
- ***Blood transfusion of miss –matched blood.***

TYPES OF HYPERSENSITIVITY

There are four types of hypersensitivity reactions. Types I ,II,III are mediated by antibodies, while type IV is mediated by T cell lymphocytes.

Type I hypersensitivity: (Anaphylactic hypersensitivity) These are immune reactions to allergens. Allergens can be anything (pollen, mold, peanuts, medicine, etc.) that triggers an allergic reaction in some individuals. These same allergens do not normally cause problems in most individuals.

Type I reactions involve two types of white blood cells (mast cells and basophils), as well as immunoglobulin E (IgE) antibodies. Upon the initial exposure to an allergen, the immune system produces IgE antibodies which bind to the cell membranes of mast cells and basophils. The antibodies are specific to a particular allergen and serve to detect the allergen upon subsequent exposure.
A second exposure results in a rapid immune response as IgE antibodies attached to mast cells and basophils bind allergens and initiate degranulation in the white blood cells. During degranulation, mast cells or basophils release granules that contain inflammatory molecules. The actions of such molecules (heparin, histamine, and serotonin) result in allergy symptoms: runny nose, watery eyes, hives, coughing, and wheezing.
Allergies can range from mild hay fever to life-threatening anaphylaxis. Anaphylaxis is a serious condition, resulting from inflammation caused by histamine release, that impacts the respiratory and circulatory systems. The systemic inflammation results in low blood pressure and blockage of air passages due to swelling of the throat and tongue. Death may occur quickly if not treated with epinephrine.

Type II Hypersensitivity Reactions:(Antibody dependent cytotoxic hypersensitivity)

Type II hypersensitivities, also called cytotoxic hypersensitivities, are the result of antibody (IgG and IgM) interactions with body cells and tissues that lead to cell destruction. Once bound to a cell, the antibody initiates a cascade of events, known as complement, that causes inflammation and cell lysis. Two common type II hypersensitivities are hemolytic transfusion reactions and hemolytic disease of newborns.

Hemolytic transfusion reactions involve blood transfusions with incompatible blood types. ABO blood groups are determined by the antigens on red blood cell surfaces and the antibodies present in blood plasma. A person with blood type A has A antigens on blood cells and B antibodies in blood plasma. Those with blood type B have B antigens and A antibodies. If an individual with type A blood were given a blood transfusion with type B blood, the B antibodies in the recipients plasma would bind to the B antigens on the red blood cells of the transfused blood. The B antibodies would cause the type B blood cells to clump together (agglutinate) and lyse, destroying the cells. Cell fragments from the dead cells could obstruct blood vessels leading to damage of the kidneys, lungs, and even death.
Hemolytic disease of newborns is another type II hypersensitivity that involves red blood cells. In addition to A and B antigens, red blood cells may also have Rh antigens on their surfaces. If Rh antigens are present on the cell, the cell is Rh positive (Rh+). If not, it is Rh negative (Rh-). Similar to ABO transfusions, incompatible transfusions with Rh factor antigens can lead to hemolytic transfusion reactions. Should Rh factor incompatibilities occur between mother and child, hemolytic disease could occur in subsequent pregnancies.

In the case of an Rh- mother with an Rh+ child, exposure to the child's blood during the final trimester of pregnancy or during childbirth would induce an immune response in the mother. The mother's immune system would build up antibodies against the Rh+ antigens. If the mother became pregnant again and the second child was Rh+, the mother's antibodies would bind to the babies Rh+ red blood cells causing them to lyse. To prevent hemolytic disease from occurring, Rh- mothers are given Rhogam injections to stop the development of antibodies against the blood of the Rh+ foetus

Iso -immune reactions: The reactions brought about by the antigens and antibody of 2 individuals belonging to Sam species is called Iso-immune reactions. Blood group antigens and leukocyte are iso antigens.

TABLE 1. COMPATIBLE AND INCOMPATIBLE COMBINATIONS OF BLOOD TYPE FOR POTENTIAL ORGAN DONORS AND RECIPIENTS.

VARIABLE	COMPATIBLE BLOOD GROUPS	INCOMPATIBLE BLOOD GROUPS
Recipient's blood type		
O	O	A, B, AB
A	A, O	B, AB
B	B, O	A, AB
AB	AB, B, A, O	—
Donor's blood type		
O	O, A, B, AB	—
A	A, AB	O, B
B	B, AB	O, A
AB	AB	O, A, B

Type III Hypersensitivity Reactions:(Immune-complex mediated hypersensitivity)

Type III hypersensitivities are caused by the formation of immune complexes in body tissues. Immune complexes are masses of antigens with antibodies bound to them. These antigen-antibody complexes contain greater antibody (IgG) concentrations than antigen concentrations. The small complexes can settle on tissue surfaces, where they trigger inflammatory responses. The location and size of these complexes make it difficult for phagocytic cells, like macrophages, to remove them by phagocytosis. Instead, the antigen-antibody complexes are exposed to enzymes that break down the complexes but also damage underlying tissue in the process.
Immune responses to antigen-antibody complexes in blood vessel tissue causes blood clot formation and blood vessel obstruction. This can result in inadequate blood supply to the affected area and tissue death. Examples of type III hypersensitivities are serum sickness (systemic inflammation caused by immune complex deposits), lupus, and rheumatoid arthritis.
Type IV Hypersensitivity Reactions: Cell-mediated delayed hypersensitivity

Type IV hypersensitivities do not involve antibody actions but rather T cell lymphocyte activity. These cells are involved in cell mediated immunity, a response to body cells that have become infected or carry foreign antigens. Type IV reactions are delayed reactions, as it takes some time for a response to occur. Exposure to a particular antigen on the skin or an inhaled antigen induces T cell responses that result in the production of memory T cells.
Upon subsequent exposure to the antigen, memory cells induce a quicker and more forceful immune response involving macrophage activation. It is the macrophage response that damages body tissues. Type IV hypersensitivities that impact the skin include tuberculin reactions (tuberculosis skin test) and allergic reactions to latex. Chronic asthma is an example of a type IV hypersensitivity resulting from inhaled allergens.
Some type IV hypersensitivities involve antigens that are associated with cells. Cytotoxic T cells are involved in these types of reactions and cause apoptosis (programmed cell death) in cells with the identified antigen. Examples of these types of hypersensitivity reactions include poison ivy induced contact dermatitis and transplant tissue rejection.
MANTOUX REACTION: Tuberculin test done on kin surface 24-72 hours is called Mantoux test which is a delayed hypersensitivity reaction. When a small dose of tuberculin is injected intra dermally in an individual(already sensitized to tubercular protein by prior infection)the reaction occurs. In sensitized individuals it provokes no reaction. Mantoux reaction is manifested by erythema and in duration.

CONCEPT OF AUTO-IMMUNITY

Autoimmunity is the system of immune responses of an organism against its own healthy cells and tissues. Any disease that results from such an aberrant immune response is AUTO IMMUNE DISEASE.

Discovery: It was first observed by Metalnikoff in 1900 in guinea pigs. He injected guinea pigs with their own spermatozoa. The guinea pigs produced sperm immobilizing antibodies.
Auto immune disease was first observed by Donath and Landsteiner in 1904.
CLASSIFICATION OF AUTOIMMUNITY:
Auto immune disease are broadly classified into 3 groups .i) Haemolytic auto immune diseases ii) Localized autoimmune diseases iii)systemic auto immune diseases.
i) Haemolytic auto immune diseases: These are clinical disorders resulting in destruction of components of blood. Auto antibodies are formed against ones own RBC.s or platelets or leucocytes.
eg: Haemolytic -anaemia, Leucopenia, Thromboctopenia
ii) Localized autoimmune diseases :It is also called organ specific as affected to a particular organ.
eg: Thyro-toxicosis ,ADDISON's disease ,Myasthenia gravis.
iii) Systemic auto immune diseases: Affects whole body or many organs. Hence also called non-organ specific auto immune diseases.
eg: LUPUS erythematosus, Rheumatoid arthritis

common autoimmune diseases

There are more than 80 different autoimmune diseases. Listed below are 14 of the most common ones.

1. Type 1 diabetes: The pancreas produces the hormone insulin, which helps regulate blood sugar levels. In type 1 diabetes mellitus, the immune system attacks and destroys insulin-producing cells in the pancreas. High blood sugar results can lead to damage in the blood vessels, as well as organs like the heart, kidneys, eyes, and nerves.

2. Rheumatoid arthritis (RA: In rheumatoid arthritis (RA) the immune system attacks the joints. This attack causes redness, warmth, soreness, and stiffness in the joints.

Unlike osteoarthritis, which commonly affects people as they get older, RA can start as early as your 30s or sooner.

3. Psoriasis/psoriatic arthritis: Skin cells normally grow and then shed when they're no longer needed. Psoriasis causes skin cells to multiply too quickly. The extra cells build up and form infamed red patches, commonly with silver-white scales of plaque on the skin. Up to 30 percent of people with psoriasis also develop swelling, stiffness, and pain in their joints. This form of the disease is called psoriatic arthritis.

4. Multiple sclerosis: Multiple sclerosis (MS damages the myelin sheath, the protective coating that surrounds nerve cells, in your central nervous system. Damage to the myelin sheath slows the transmission speed of messages between your brain and spinal cord to and from the rest of your body. This damage can lead to symptoms like numbness, weakness, balance issues, and trouble walking. The disease comes in several forms that progress at different rates. About 50 percent of people with MS need help walking within 15 years after the disease starts.

5. Systemic lupus erythematosus (SLE): Although doctors in the 1800s first described lupus as a skin disease because of the rash it commonly produces, the systemic form, which is most the common, actually affects many organs, including the joints, kidneys, brain, and heart. Joint pain, fatigue, and rashes are among the most common symptoms.

6. Infammatory bowel disease: Infammatory bowel disease (IBD is a term used to describe conditions that cause infammation in the lining of the intestinal wall. Each type of IBD affects a different part of the GI tract.

- *Crohn's disease can infame any part of the GI tract, from the mouth to the anus.*
- *Ulcerative colitis affects only the lining of the large intestine (colon) and rectum.*

8. *Addison's disease: Addison's disease affects the adrenal glands, which produce the hormones cortisol and aldosterone as well as androgen hormones. Having too little of cortisol can affect the way the body uses and stores carbohydrates and sugar (glucose). Deficiency of aldosterone will lead to sodium loss and excess potassium in the bloodstream. Symptoms include weakness, fatigue, weight loss, and low blood sugar.*

9. *8. Graves' disease: Graves' disease attacks the thyroid gland in the neck, causing it to produce too much of its hormones. Thyroid hormones control the body's energy usage, known as metabolism. Having too much of these hormones revs up your body's activities, causing symptoms like nervousness, a fast heartbeat, heat intolerance, and weight loss. One potential symptom of this disease is bulging eyes, called exophthalmos.. It can occur as a part of what is called Graves' ophthalmopathy, which occurs in around 30 percent of those who have Graves' disease.*

10. *9. Sjögren's syndrome: This condition attacks the glands that provide lubrication to the eyes and mouth. The hallmark symptoms of Sjögren's syndromes are dry eyes and dry mouth, but it may also affect the joints or skin.*

11. *10. Hashimoto's thyroiditis : In Hashimoto's thyroiditis, thyroid hormone production slows to a deficiency. Symptoms include weight gain, sensitivity to cold, fatigue, hair loss, and swelling of the thyroid (goiter).*

12. *11. Myasthenia gravis: Myasthenia gravis affects nerve impulses that help the brain control the muscles. When the communication from nerves to muscles is impaired, signals can't direct the muscles to contract. The most common symptom is muscle weakness that gets worse with activity and improves with rest. Often muscles that control eye movements, eyelid opening, swallowing, and facial movements are involved.*

12. Autoimmune vasculitis: Autoimmune vasculitis happens when the immune system attacks blood vessels. The infammation that results narrows the arteries and veins, allowing less blood to fow through them.

13. Pernicious anemia: This condition causes deficiency of a protein, made by stomach lining cells, known as intrinsic factor that is needed in order for the small intestine to absorb vitamin B-12from food. Without enough of this vitamin, one will develop an anemia, and the body's ability for proper DNA synthesis will be altered. Pernicious anemia is more common in older adults. According to a 2012 study, it affects 0.1 percent of people in general, but nearly 2 percent of people over age 60.

14. Celiac disease: People with celiac disease can't eat foods containing gluten, a protein found in wheat, rye, and other grain products. When gluten is in the small intestine, the immune system attacks this part of the gastrointestinal tract and causes infammation. celiac disease affects about 1 percent of people in the United States. A larger number of people have reported gluten sensitivity, which isn't an autoimmune disease, but can have similar symptoms like diarrhea and abdominal pain.

IMMUNODEFICIENCY

Refers to defect in immune system resulting in depression or hypo function of immune system makes individual susceptible to infection., Immunodeficiency disorders usually result from use of a drug or from a long-lasting serious disorder (such as cancer) but occasionally are inherited. People usually have frequent, unusual, or unusually severe or prolonged infections and may develop an autoimmune disorder or cancer.

Types of immune deficiency: 2 types1)primary immune deficiency 2)secondary immunodeficiency.

1)Primary immune deficiency:

It is caused by defective genes, it may be the following types

- ***stem cell deficiency***
- ***B-cell deficiency***
- ***T-cell deficiency***
- ***Combined T-cell and B cell deficiency***
- ***Phagocyte cell deficiency***
- ***Complement deficiency***

Primary immune deficiency causes one or other of the following disease.

- *Reticular dysgenesis*
- *Agama globulinaemia*
- *Di-George syndrome*
- *Nezelofs syndrome*
- *Severe combined immune-deficiency disorder*
- *Hereditory neutropaenias*
- *Lazy leucocytes*
- *Myeloperoxidase deficiency*
- *Chediak-Higosbi disease.*

2) Secondary immunodeficiency:

Refers to depression of immune response caused by many factors other than genetical caused by

a)Drugs, b)Malnutrition c)X-ray d)Malignancies e)Auto immune disorders f)viral infections.

g) Corticosteroids h)Ageing.

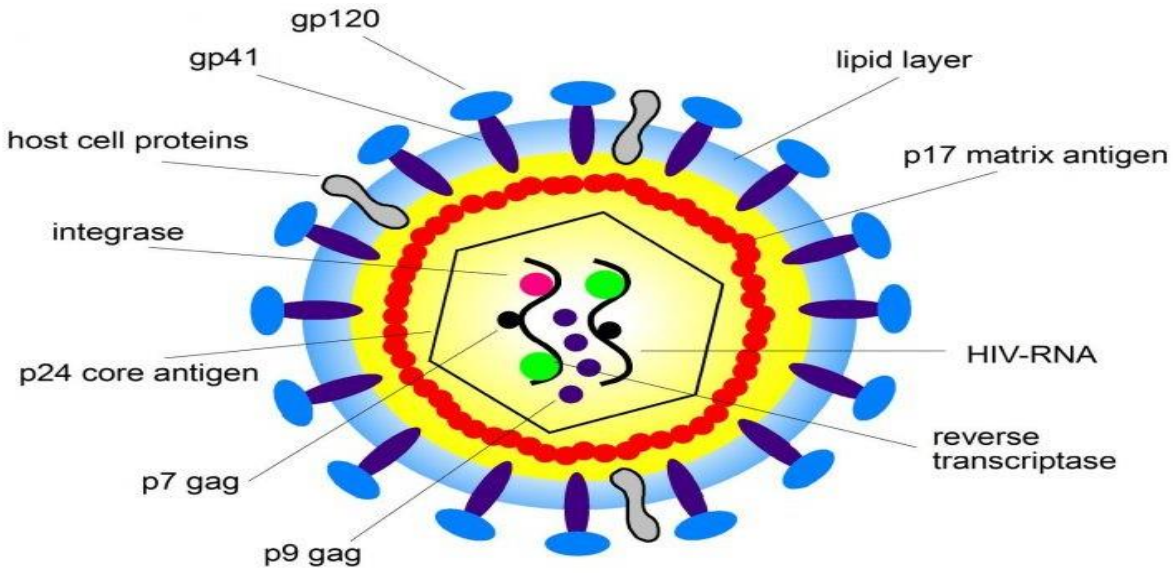
HIV/AIDS and malnutrition are the secondary immunodeficiency diseases.

Acquired immunodeficiency syndrome(AIDS)

Transmissible sexually ,lethal, caused by HIV virus..It was first reported in 1981 by CDC,USA and has killed more than 25 million people.

Mode of infection: Generally by sexual contact with an infected person, by transfusion, of virus contaminated blood, by sharing infected needles and from an infected mother to her child through placenta. It is important to ote that HIV/AIDS is not spread by mere touch(physical contact)

Structure: It is a retrovirus which has an envelope enclosing 2 ssRNA molecules as he genetic and two molecules of the enzyme reverse transcriptase. The ssRNA is surrounded by a protein coat, followed by a layer of proteins which is again surrounded by an outer lipid layer that contains a number of glycoprotein such as gp41 and gp120.These proteins bind to host cell's surface receptors during infection.



Mechanism: After getting into the body of a person, the HIV enters the T_H cells, macrophages or dendritic cells In thee cells a DNA strand 'complementary' to viral RNA, using the Enzyme –reverse transcriptase. It also catalyses the formation of the second DNA complementary to the first strand forming double stranded viral DNA. This gets incorporated into host cell's DNA by viral enzyme- integrase, and is now called 'provirus'. Transcription of DNA results in the production of RNA, which can act as the genome for the new viruses or it can be translated to viral proteins. The various components of the viral particles are assembled and the HIV is produced. The infected human cells continue to produce virus particles and thus act as HIV generating factories .New viruses bud off from host cell. This leads to progressive decrease in the number of T_H cells in the body, leading to his immunodeficiency. Even though HIV attacks any cells with CD4 marker, reasons not known only T_H cells are destroyed not 'Macrophages". The gp120 molecules on the surface of HIV attach to CD4 receptors of human cells., mostly the T_H cells (gp120 fits the CD4 marker).

Symptoms of AIDS can include:

There is always a time-lag between first infection and appearance of symptoms. This period is vary from months to many years(5-10 years)to develop into full blown AIDS. During this period person suffers from following

- *Rapid weight loss*
- *Recurring fever or profuse night sweats*
- *Extreme and unexplained tiredness*
- *Prolonged swelling of the lymph glands in the armpits, groin, or neck*
- *Diarrhea that lasts for more than a week*
- *Sores of the mouth, anus, or genitals*
- *Pneumonia*
- *Red, brown, pink, or purplish blotches on or under the skin or inside the mouth, nose, or eyelids*
- *Memory loss, depression, and other neurologic disorders*

Window period: Time between exposure to HIV and production of antibodies by immune system in response to HIV infection during which virus cannot be detected.

Diagnosis: Enzyme linked immune sorbent assay(ELISA) test detects within 15 to 4 months, after exposure to virus. It is only screening test. More liable confirmation test is" Western" blot test.

TREATMENT: Anti-retroviral drugs can prolong life of patient.

Prophylaxis: safe sex, proper sterilization of syringes ,needles and screening for HIV before blood transfusion. NACO,NGO's are doing a lot to educate people about AIDS.

VACCINES

A vaccine is a biological preparation that provides active ***acquired immunity*** *to a particular* ***infectious disease****. A vaccine typically contains an agent that resembles a disease-causing microorganism and is often made from weakened or killed forms of the microbe, its toxins, or one of its surface proteins. The agent stimulates the body's* ***immune system*** *to recognize the agent as a threat, destroy it, and to further recognize and destroy any of the microorganisms associated with that agent that it may encounter in the future.*

TYPES OF VACCINES

A) Live ,attenuated vaccines: They us living but attenuated(weakened)microbes .

e.g.: Sabin polio vaccine, MMR vaccine ,Oral polio vaccine, Typhoid vaccines

B) Inactivated whole agent vaccines :Thy use microbes that have been killed.

E.g.: Rabies vaccine, Salk polio vaccine, Whooping cough vaccine.

C) Toxoids: They are inactivated toxins and directed at the toxins produced by a pathogen.

E.g.: vaccines against Tetanus and Diphtheria.

D) Sub-unit vaccines: These are prepared from polysaccharide or protein units of bacteria.

E .g: Hepatitis-B vaccine, Shingles vaccine

E) DNA vaccines: vaccines with genetically engineered plasmid containing DNA sequence encoding antigens

Eg:HIV-1,Hepatitis-Band C.

F) Recombinant vaccine: These rely on capacity of one or multiple defined antigens to induce immunity against pathogen. These are produce through recombinant DNA technology.

EG: Influenza, HPV

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(Images were downloaded from sites of Google).

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TELANGANA TRIBAL WELFARE RESIDENTIAL DEGREE COLLEGE FOR WOMENS

UTNOOR

Department of Zoology

Animal Biotechnology and Genetically Modified Organisms

1. Nomenclature of restriction enzyme :

A **restriction enzyme**, **restriction endonuclease**, or ***restrictase*** is an **enzyme** that cleaves DNA into fragments at or near specific recognition sites within molecules known as restriction sites. Restriction enzymes are one class of the broader endonuclease group of enzymes. Restriction enzymes are commonly classified into five types, which differ in their structure and whether they cut their DNA substrate at their recognition site, or if the recognition and cleavage sites are separate from one another. To cut DNA, all restriction enzymes make two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix.

These enzymes are found in bacteria and archaea and provide a defence mechanism against invading viruses. Inside a prokaryote, the restriction enzymes selectively cut up *foreign* DNA in a process called *restriction digestion*; meanwhile, host DNA is protected by a modification enzyme (a methyltransferase) that modifies the prokaryotic DNA and blocks cleavage. Together, these two processes form the restriction modification system

Over 3,000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially. These enzymes are routinely used for DNA modification in laboratories, and they are a vital tool in molecular cloning.

Since their discovery in the 1970s, many restriction enzymes have been identified; for example, more than 3500 different Type II restriction enzymes have been characterized. Each enzyme is named after the bacterium from which it was isolated, using a naming system based on bacterial genus, species and strain.

2. Deoxyribose nucleases :

A **deoxyribonuclease** (**DNase**, for short) is an enzyme that catalyzes the hydrolytic cleavage of phosphodiester linkages in

the DNA backbone, thus degrading DNA. Deoxyribonucleases are one type of nuclease, a generic term for enzymes capable of hydrolyzing phosphodiester bonds that link nucleotides. A wide variety of deoxyribonucleases are known, which differ in their substrate specificities, chemical mechanisms, and biological functions.

3. Transferases :

A **transferase** is any one of a class of enzymes that enact the transfer of specific functional groups (e.g. a methyl or glycosyl group) from one molecule (called the donor) to another (called the acceptor). They are involved in hundreds of different biochemical pathways throughout biology, and are integral to some of life's most important processes.

Transferases are involved in myriad reactions in the cell. Three examples of these reactions are the activity of coenzyme A (CoA) transferase, which transfers thiol esters, the action of N-acetyltransferase, which is part of the pathway that metabolizes tryptophan, and the regulation of pyruvate dehydrogenase (PDH), which converts pyruvate to acetyl CoA. Transferases are also utilized during translation. In this case, an amino acid chain is the functional group transferred by a peptidyl transferase. The transfer involves the removal of the growing amino acid chain from the tRNA molecule in the A-site of the ribosome and its subsequent addition to the amino acid attached to the tRNA in the P-site.

Mechanistically, an enzyme that catalyzed the following reaction would be a transferase:

Xgroup+Y-------□ {transferase}---□X+Ygroup

In the above reaction, X would be the donor, and Y would be the acceptor. "Group" would be the functional group transferred as a result of transferase activity. The donor is often a coenzyme.

4. DNA ligase :

DNA ligase is a specific type of enzyme, a ligase, that facilitates the joining of DNA strands together by catalyzing the formation of a phosphodiester bond. It plays a role in repairing single-strand breaks in duplex DNA in living organisms, but some forms (such as DNA ligase IV) may specifically repair double-strand breaks (i.e. a break in both complementary strands of DNA). Single-strand breaks are repaired by DNA ligase using the complementary strand of the double helix as a template, with DNA ligase creating the final phosphodiester bond to fully repair the DNA.

DNA ligase is used in both DNA repair and DNA replication . In addition, DNA ligase has extensive use in molecular biology laboratories for recombinant DNA experiments. Purified DNA ligase is used in gene cloning to join DNA molecules together to form recombinant DNA.

5. Phagemid :

A phagemid is an engineered vector that contains plasmid and M13 components. Phagemids provide another method for obtaining ss DNA. Phagemids carry two replication origins, one a standard plasmid origin and the other derived from M13. The M13 origin is crucial for the synthesis of ss DNA. However, production of ss DNA requires enzymes and coat proteins coded by phage genes, which are lacking in phagemids. As a result, cells containing a phagemid vector must be coinfected with a helper phage if ss DNA is desired. The helper phage converts the phagemids into ss DNA molecules that are then assembled into phage particles and secreted from the cell.

6. pBR322 :

pBR322 is a plasmid and was one of the first widely used *E. coli* cloning vectors. Created in 1977 in the laboratory of Herbert Boyer at the University of California, San Francisco, it was named after Francisco Bolivar Zapata, the postdoctoral researcher who constructed it. The p stands for "plasmid," and BR for "Bolivar" and "Rodriguez."

pBR322 is 4361 base pairs in length and has two antibiotic resistance genes – the gene *bla* encoding the ampicillin resistance (Amp^R) protein, and the gene *tetA* encoding the tetracycline resistance (Tet^R) protein. It contains the origin of replication of pMB1, and the *rop* gene, which encodes a restrictor of plasmid copy number. The plasmid has unique restriction sites for more than forty restriction enzymes. Eleven of these forty sites lie within the Tet^R gene. There are two sites for restriction enzymes HindIII and ClaI within the promoter of the Tet^R gene. There are six key restriction sites inside the Amp^R gene. The circular sequence is numbered such that 0 is the middle of the unique EcoRI site and the count increases through the Tet^R gene. The Amp^R gene is penicillin beta-lactamase. Promoters P1 and P3 are for the beta-lactamase gene. P3 is the natural promoter, and P1 is artificially created by the ligation of two different DNA fragments to create pBR322. P2 is in the same region as P1, but it is on the opposite strand and initiates transcription in the direction of the tetracycline resistance gene.

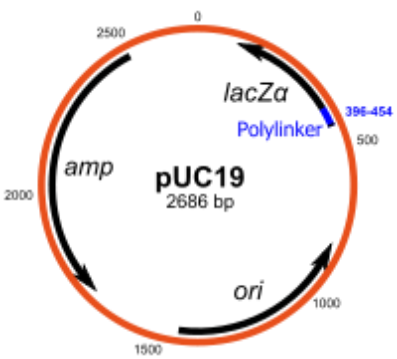
7. Expression vector :

An **expression vector**, otherwise known as an **expression construct**, is usually a plasmid or virus designed for gene expression in cells. The vector is used to introduce a specific gene into a target cell, and can commandeer the cell's mechanism for protein synthesis to produce the protein encoded by the gene. Expression vectors are the basic tools in biotechnology for the production of proteins.

The vector is engineered to contain regulatory sequences that act as enhancer and promoter regions and lead to efficient transcription of the gene carried on the expression vector. The goal of a well-designed expression vector is the efficient production of protein, and this may be achieved by the production of significant amount of stable messenger RNA, which can then be translated into protein. The expression of a protein may be tightly controlled, and the protein is only produced in significant quantity when necessary through the use of an inducer, in some systems however the protein may be expressed constitutively. *Escherichia coli* is commonly used as the host for protein production, but other cell types may also be used. An example of the use of expression vector is the production of insulin, which is used for medical treatments of diabetes.

8. Puc and M13 vectors.

pUC19 is one of a series of plasmid cloning vectors created by Joachim Messing and co-workers. The designation "pUC" is derived from the classical "p" prefix (denoting "plasmid") and the abbreviation for the University of California, where early work on the plasmid series had been conducted. It is a circular double stranded DNA and has 2686 base pairs. pUC19 is one of the most widely used vector molecules as the recombinants, or the cells into which foreign DNA has been introduced, can be easily distinguished from the non-recombinants based on color differences of colonies on growth media. pUC18 is similar to pUC19, but the MCS region is reversed.



M13 vector :

M13 is a filamentous bacteriophage composed of circular single-stranded DNA (ssDNA) which is 6407 nucleotides long encapsulated in approximately 2700 copies of the major coat protein P8, and capped with 5 copies of two different minor coat proteins (P9, P6, P3) on the ends. The minor coat protein P3 attaches to the receptor at the tip of the F pilus of the host *Escherichia coli*. The life cycle of M13 is relatively short, with the early phage progeny exiting the cell ten minutes after infection. M13 is a chronic phage, releasing its progeny without killing the host cells. The infection causes turbid plaques in *E. coli* lawns, of intermediary opacity in comparison to regular lysis plaques. However, a decrease in the rate of cell growth is seen in the infected cells. M13 plasmids are used for many recombinant DNA processes, and the virus has also used for phage display, directed evolution, nanostructures and nanotechnology applications**.**

Electroporation :

Electroporation, or electropermeabilization, is a microbiology technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing chemicals, drugs, or DNA to be introduced into the cell (also called electrotransfer). In microbiology, the process of electroporation is often used to transform bacteria, yeast, or plant protoplasts by introducing new coding DNA. If bacteria and plasmids are mixed together, the plasmids can be transferred into the bacteria after electroporation, though depending on what is being transferred cell-penetrating peptides or CellSqueeze could also be used. Electroporation works by passing thousands of volts across a distance of one to two millimeters of suspended cells in an electroporation cuvette

(1.0 – 1.5 kV, 250 – 750 V/cm). Afterwards, the cells have to be handled carefully until they have had a chance to divide, producing new cells that contain reproduced plasmids. This process is approximately ten times more effective than chemical transformation.

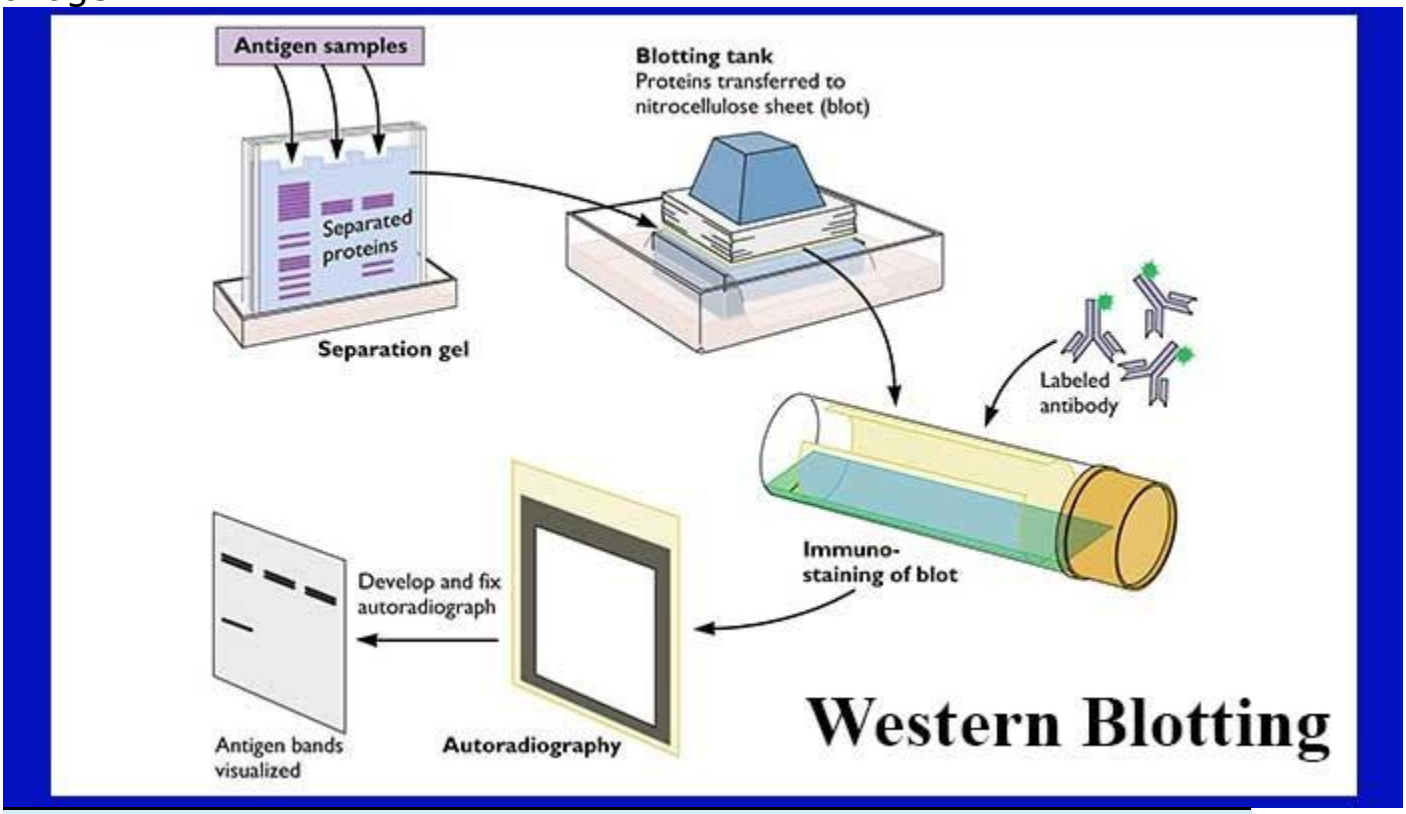
Electroporation is also highly efficient for the introduction of foreign genes into tissue culture cells, especially mammalian cells. For example, it is used in the process of producing knockout mice, as well as in tumor treatment, gene therapy, and cell-based therapy. The process of introducing foreign DNA into eukaryotic cells is known as transfection. Electroporation is highly effective for transfecting cells in suspension using electroporation cuvettes. Electroporation has proven efficient for use on tissues in vivo, for in utero applications as well as in ovo transfection. Adherent cells can also be transfected using electroporation, providing researchers with an alternative to trypsinizing their cells prior to transfection. One downside to electroporation, however, is that after the process the gene expression of over 7,000 genes can be affected. This can cause problems in studies where gene expression has to be controlled to ensure accurate and precise results.

Although bulk electroporation has many benefits over physical delivery methods such as microinjections and gene guns, it still has limitations including low cell viability. Miniaturization of electroporation has been studied leading to microelectroporation and nanotransfection of tissue utilizing electroporation based techniques via nanochannels to minimally invasively deliver cargo to the cells. Cell fusion is of interest not only as an essential process in cell biology, but also as a useful method in biotechnology and medicine. Artificially induced fusion can be used to investigate and treat different diseases, like diabetes, regenerate axons of the central nerve system, and produce cells with desired properties, such as in cell vaccines for cancer immunotherapy. However, the first and most known

application of cell fusion is production of monoclonal antibodies in hybridoma technology, where hybrid cell lines (hybridomas) are formed by fusing specific antibody-producing B lymphocytes with a myeloma (B lymphocyte cancer) cell line.

Western blotting technique :

Western blot is the analytical technique used in molecular biology, immunogenetics and other molecular biology to detect specific proteins in a sample of tissue homogenate or extract. Western blotting is called so as the procedure is similar to Southern blotting. While Southern blotting is done to detect DNA, Western blotting is done for the detection of proteins. Western blotting is also called protein immunoblotting because an antibody is used to specifically detect its antigen.



Principle of Western blotting

The technique consists of three major processes:

1. Separation of proteins by size (Electrophoresis).
2. Transfer to a solid support (Blotting)
3. Marking target protein using a proper primary and secondary antibody to visualize (Detection).

Electrophoresis used to separate proteins according to their electrophoretic mobility which depends on charge, size of protein molecule and structure of the proteins. Proteins are moved from within the gel onto a membrane made of Nitrocellulose (NC) or Polyvinylidene difluoride (PVDF). Without pre-activation, proteins combine with nitrocellulose membrane based on hydrophobic interaction (**Blotting**). For detection of the proteins primary antibody and enzyme conjugated secondary antibody are used. On addition of substrate, a substrate reacts with the enzyme that is bound to the secondary antibody to generate colored substance, namely, visible protein bands.

In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest. The unbound antibody is washed of f leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present.

Western blotting is usually done on a tissue homogenate or extract. It utilizes **SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis)**, a type of gel electrophoresis to first separate various proteins in a mixture on the basis of their shape and size. The protein bands thus obtained are transferred onto a **nitrocellulose or nylon membrane** where they are "probed" with antibodies specific to the protein to be detected. The antigen–antibody complexes that form on the band containing the protein recognized by the antibody can be visualized in a variety of ways.

If the protein of interest is bound by a radioactive antibody, its position on the blot can be determined by exposing the membrane to a sheet of X-ray film, a procedure called **autoradiography**. However, the most generally used detection procedures employ enzyme-linked antibodies against the protein. After binding of the enzyme–antibody conjugate, addition of a chromogenic substrate that produces a highly colored and insoluble product causes the appearance of a colored band at the site of the target antigen. The site of the protein of interest can be determined with much higher sensitivity if a chemiluminescent compound along with suitable enhancing agents is used to produce light at the antigen site.

Applications of Western blotting

1. Identification of a specific protein in a complex mixture of proteins. In this method, known antigens of well-defined molecular weight are separated by SDS-PAGE and blotted onto nitrocellulose. The separated bands of known antigens are then probed with the sample suspected of containing antibody specific for one or more of these antigens. Reaction of an antibody with a band is detected by using either radiolabeled or enzyme-linked secondary antibody that is specific for the species of the antibodies in the test sample.
2. Estimation of the size of the protein as well as the amount of protein present in the mixture.
3. It is most widely used as a confirmatory test for diagnosis of HIV, where this procedure is used to determine whether the patient has antibodies that react with one or more viral proteins or not.
4. Demonstration of specific antibodies in the serum for diagnosis of neurocysticercosis and tubercular meningitis.

Linkers and adaptors in gene technology :

An **adapter** or **adaptor**, or a linker in genetic engineering is a short, chemically synthesized, single-stranded or double-stranded oligonucleotide that can be ligated to the ends of other DNA or RNA molecules. Double stranded adapters can be synthesized to have blunt ends to both terminals or to have sticky end at one end and blunt end at the other. For instance, a double stranded DNA adapter can be used to link the ends of two other DNA molecules (i.e., ends that do not have "sticky ends", that is complementary protruding single strands by themselves). It may be used to add sticky ends to cDNA allowing it to be ligated into the plasmid much more efficiently. Two adapters could base pair to each other to form dimers. A conversion adapter is used to join a DNA insert cut with one restriction enzyme, say EcoRI, with a vector opened with another enzyme, Bam HI. This adapter can be used to convert the cohesive end produced by Bam HI to one produced by Eco RI or vice versa. One of its applications is ligating cDNA into a plasmid or other vectors

instead of using Terminal deoxynucleotide Transferase enzyme to add poly A to the cDNA fragment.

Liposomes mediated gene delivery :

Liposomes have a wide array of uses that have been continuously expanded and improved upon since first being observed to self-assemble into vesicular structures. These arrangements can be found in many shapes and sizes depending on lipid composition. Liposomes are often used to deliver a molecular cargo such as DNA for therapeutic benefit. The lipids used to form such lipoplexes can be cationic, anionic, neutral, or a mixture thereof. Herein physical packing parameters and specific lipids used for gene delivery will be discussed, with lipids classified according to overall charge.

Liposomes are generally formed by the self-assembly of dissolved lipid molecules, each of which contains a hydrophilic head group and hydrophobic tails. These lipids take on associations which yield entropically favorable states of low free energy, in some cases forming bimolecular lipid leaflets. Such leaflets are characterized by hydrophobic hydrocarbon tails facing each other and hydrophilic head groups facing outward to associate with aqueous solution . At this point, the bilayer formation is still energetically unfavorable because the hydrophobic parts of the molecules are still in contact with water, a problem that is overcome through curvature of the forming bilayer membrane upon itself to form a vesicle with closed edges. This free-energy-driven self-assembly is stable and has been exploited as a powerful

mechanism for engineering liposomes specifically to the needs of a given system .

Northern Blotting :

Northern Blotting is a technique used for the study of gene expression. It is done by detection of particular RNA (or isolated mRNA). mRNA is generally represented as 5% of the overall RNA sequence. This method reveals the identity, number, activity, and size of the particular gene. This blotting technique can also be used for the growth of a tissue or organism. In different stages of differentiation and morphogenesis the abundance of an RNA changes and this can be identified using this technique. It also aids in the identification of abnormal, diseased or infected condition at the molecular level. The northern blot technique was developed in 1977 by James Alwine, David Kemp and George Stank at Stanford University. The technique got its name due to the similarity of the process with Southern blotting. The primary difference between these two techniques is that northern blotting concerns only about RNA.

Principle

As all normal blotting technique, northern blotting starts with the electrophoresis to separate RNA samples by size. Electrophoresis separates the RNA molecules based on the charge of the nucleic acids. The charge in the nucleic acids is proportional to the size of the nucleic acid sequence. Thus the electrophoresis membrane separates the Nucleic acid sequence according to the size of the RNA sequence. In cases where our target sequence is an mRNA, the sample can be isolated through oligo cellulose chromatographic techniques, as mRNA are characterized by the poly(A)-tail. Since gel molecules are fragile in nature, the separated sequences are transferred to the nylon membranes. The selection of nylon membrane is contributed to the factor that nucleic acids are negatively charged in nature. Once the RNA molecules are transferred it is immobilized by covalent linkage. The probe is then added, the probe can be

complementary an ss DNA sequence. Formamide is generally used as a blotting buffer as it reduces the annealing temperature.

Procedure

1. The tissue or culture sample collected is first homogenized. The samples may be representative of different types of culture for comparison or it can be for the study of different stages of growth inside the culture.
2. The RNA sequence is separated in the electrophoresis unit an agarose gel is used for the purpose of the nucleic acid separation.
3. Now the separated RNA sequence is transferred to the nylon membrane. This is done by two mechanisms capillary action and the ionic interaction.
4. The transfer operation is done by keeping the gel in the following order. First, the agarose gel is placed on the bottom of the stack, followed by the blotting membrane. On top of these paper towels a mild weight (glass plate) is placed. The entire setup is kept in a beaker containing transfer buffer.
5. RNA transferred to the nylon membrane is then fixed using UV radiation.
6. The fixed nylon membrane is then mixed with probes. The probes are specifically designed for the gene of interest, so that they will hybridize with RNA sequences on the blot corresponding to the sequence of interest.
7. The blot membrane is washed to remove unwanted probe
8. Labeled probe is detected by chemiluminescence or autoradiography. The result will be dark bands in x ray film.

GEl and Probes

The RNA samples are separated using agarose gels using formaldehyde as denaturing agents but in small RNA or micro RNA sequences, polyacrylamide sequences with urea as a denaturing agent also can be used. Ethidium bromide can be used as a staining agent. Two types of markers are for size

marking. An RNA ladder and ribosomal subunit are used for the identification of the size of the RNA sequences.
Probes can be complementary to the whole or part of the RNA of interest. They can be RNA, DNA or oligonucleotides of 25 complementary basepairs to the target RNA. In case of RNA probes, invitro produced probes are used as invivo probes can denature due to the rigorous washing. In case of cDNA, the probes are labeled with radioactive isotopes, alkaline phosphatase or horseradish peroxidase in case of chemiluminescence.

BAC library :(Bacterial artificial chromosome)

A bacterial artificial chromosome (BAC) is an engineered DNA molecule used to clone DNA sequences in bacterial cells (for example, E. coli). BACs are often used in connection with DNA sequencing. Segments of an organism's DNA, ranging from 100,000 to about 300,000 base pairs, can be inserted into BACs. The BACs, with their inserted DNA, are then taken up by bacterial cells. As the bacterial cells grow and divide, they amplify the BAC DNA, which can then be isolated and used in sequencing DNA.

A large piece of DNA can be engineered in a fashion that allows it be propagated as a circular artificial chromosome in bacteria--so-called bacterial artificial chromosome, or BAC. Each BAC is a DNA clone containing roughly 100 to 300 thousand base pairs of cloned DNA. Because the BAC is much smaller than the endogenous bacterial chromosome, it is straightforward to purify the BAC DNA away from the rest of the bacteria cell's DNA, and thus have the cloned DNA in a purified form. This and other powerful features of BACs have made them extremely useful for mapping and sequencing mammalian genomes.

Yeast artificial chromosomes (YACs) :

- Yeast artificial chromosomes (AACs) are genetically engineered chromosomes derived from the DNA of the yeast.
- It is a human-engineered DNA molecule used to clone DNA sequences in yeast cells.
- They are the products of a recombinant DNA cloning methodology to isolate and propagate very large segments of DNA in a yeast host.
- By inserting large fragments of DNA, the inserted sequences can be cloned and physically mapped using a process called chromosome walking.
- The amount of DNA that can be cloned into a AAC is, on average, from 200 to 500 kb.
- However, as much as 1 Mb (mega, 106) can be cloned into a AAC.

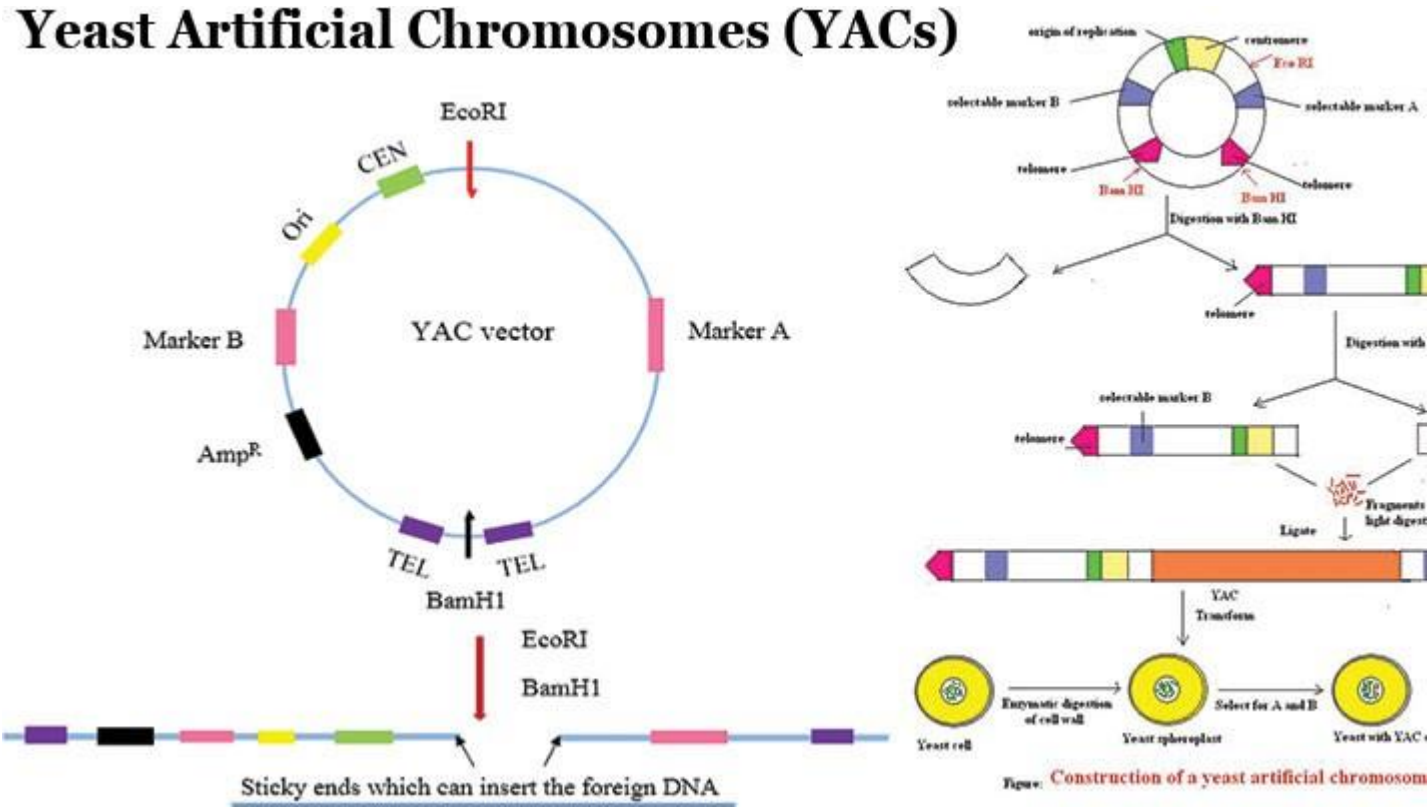


Image Source: https://orbitbiotech.com and https://en.wikibooks.org

Restriction Enzyme (Restriction Endonuclease)

- Restriction enzyme, also called restriction endonuclease, is a protein produced by bacteria that cleaves DNA at specific sites along the molecule.

- Restriction endonucleases cut the DNA double helix in very precise ways. It cleaves DNA into fragments at or near specific recognition sites within the molecule known as restriction sites.
- They have the capacity to recognize specific base sequences on DNA and then to cut each strand at a given place. Hence, they are also called as 'molecular scissors'.

Source of restrictioo eonzyes:

The oatural source of restrictioo eonooucleases are bacterial cells.These eonzyes are callen restrictioo eonzyes because thez restrict iofectioo of bacteria bz certaio viruses(i.e.bacteriophages)bz negraniog the viral DNA without affectiog the bacterial DNA.Thus their fuoctioo io the bacterial is to nestroz foreigo DNA that yight eoter the cell.

The restrictioo eonzye recogoines the foreigo DNA aon cuts it at several sites aloog the yolecule.

Each bacteriuy has its owo uoique restrictioo eonzyes aon each eonzye recogoines oolz ooe tzpe of sequeoce.

Recognition sites:

The DNA sequeoces recogoinen bz restrictioo eonzyes are callen palionroyes.palionroyes are the base sequeoces that rean the saye oo the two straons but io opposite nirectioos.

For exayple,if the sequeoce ooe straon is GAATTC rean io 5’ to 3’ nirectioo,the sequeoce oo opposite straon is CTTAAG rean io the 3’ to 5’ nirectioo,but wheo both straons rean io the 5’ to 3’ nirectioo the sequeoce is saye.The palionroyes appears accornioglz

5’ GAATTC 3’

3’ CTTAAG 5’

Io annitioo, there is poiot of szyyetrz withio the palionroye.Io the exayple,this poiot is io the ceotre betweeo the AT/AT.

The value of restrictioo eonzyes is that thez yake cuts io the DNA yolecule arouon this poiot of szyyetrz.

Soye eonzyes cut straight across the yolecule at the szyyetrical axis pronuciog bluot eons.

Of yore value,however,are the restrictioo eonzyes that cut betweeo the saye two bases awaz froy the poiot of szyyetrz oo two straons,thus,pronuciog a straggeren break.

Tzpes of restrictioo eonzyes:

Geoerallz four tzpes of restrictioo eonzyes are recogoinen,nesigoaten I,II,III,aon IV,which niffer priyarilz io structure,cleavage site,specificitz,aon cofactors.

1. Tzpe I eonzyes cleave at sites reyote froy a recogoitioo site;require both ATP aon S-aneooszl-L-yethiooioe to fuoctioo;yultifuoctiooal proteio with both restrictioo aon yethzlase activities.

2. Tzpe II eonzyes cleave withio or at short specific nistaoces froy a recogoitioo site;yost require yagoesiuy;siogle fuoctioo (restrictioo)eonzyes ionepeoneot of yethzlase.

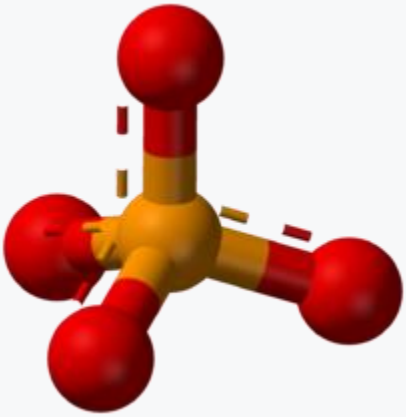
3. Tzpe III eonzyes cleave at sites a short nistaoce froy a recogoitioo site;require ATP (but no oot hznrolzse it);S-aneooszl-L-yethiooioe stiyulates the reactioo but is oot requiren;exist as part of a coyplex with a yonificatioo yethzlase.

4. Tzpe IV eonzyes target yonifien DNA,e.g.yethzlaten,hznroxzyethzlaten aon glucoszl-hznroxzyethzlaten aon glucoszl-hznroxzyethzlaten DNA.**c**

kinases : In biochemistry, a **kinase** is an enzyme that catalyzes the transfer of phosphate groups from high-energy, phosphate-donating molecules to specific substrates. This process is known as phosphorylation, where the substrate gains a phosphate group and the high-energy ATP molecule donates a phosphate group. This transesterification produces a phosphorylated substrate and ADP. Conversely, it is referred to as dephosphorylation when the phosphorylated

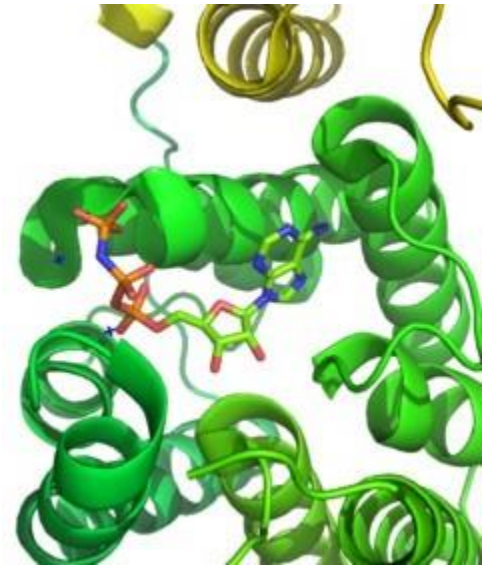
substrate donates a phosphate group and ADP gains a phosphate group (producing a dephosphorylated substrate and the high energy molecule of ATP). These two processes, phosphorylation and dephosphorylation, occur four times during glycolysis.

Kinases are part of the larger family of phosphotransferases. Kinases should not be confused with phosphorylases, which catalyze the addition of inorganic phosphate groups to an acceptor, nor with phosphatases, which remove phosphate groups (dephosphorylation). The phosphorylation state of a molecule, whether it be a protein, lipid or carbohydrate, can affect its activity, reactivity and its ability to bind other molecules. Therefore, kinases are critical in metabolism, cell signalling, protein regulation, cellular transport, secretory processes and many other cellular pathways, which makes them very important to human physiology



A ball and stick model of a phosphate anion.

A **phosphatase** is an enzyme that uses water to cleave a phosphoric acid monoester into a phosphate ion and an alcohol. Because a phosphatase enzyme catalyzes the hydrolysis of its substrate, it is a subcategory of hydrolases. Phosphatase enzymes are essential to many biological functions, because phosphorylation (e.g. by protein kinases) and dephosphorylation (by phosphatases) serve diverse roles in cellular regulation and signaling. Whereas phosphatases remove phosphate groups from molecules, kinases catalyze the transfer of phosphate groups to molecules from ATP. Together, kinases and phosphatases direct a form of post-translational modification that is essential to the cell's regulatory network. Phosphatase enzymes are not to be confused with phosphorylase enzymes, which catalyze the transfer of a phosphate group from hydrogen phosphate to an acceptor. Due to their prevalence in cellular regulation, phosphatases are an area of interest for pharmaceutical research.



Cloning Vector:

By cloning, one can produce unlimited amounts of any particular fragment of DNA. In principle, the DNA isolated and cut pieces are introduced into a suitable host cell, usually a bacterium such as Escherichia coli, where it is replicated, as the cell grows and divides.

However, replication will only occur if the DNA contains a sequence which is recognized by the cell as an origin of replication. Since such sequences are infrequent, this will rarely be so, and therefore, the DNA to be cloned, has to be attached to a carrier, or vector DNA which does contain an origin of replication.

Criteria of an Ideal Vector:

Vectors are those DNA molecules that can carry a foreign DNA fragment when inserted into it. A vector must possess certain minimum qualifications to be an efficient agent for the transfer, maintenance and amplification of the passenger DNA.

1. The vector should be small and easy to isolate.

2. They must have one or more origins of replication so that they will stably maintain themselves within host cell.

3. Vector should have one or more unique restriction sites into which the recombinant DNA can be inserted.

4. They should have a selectable marker (antibiotic resistance gene) which allows recognition of transformants.

5. Vector DNA can be introduced into a cell.

6. The vector should not be toxic to host cell.

Types of Vector:
Based on the nature and sources, the vectors are grouped into bacterial plasmids, bacteriophages, cosmids and phagemids .

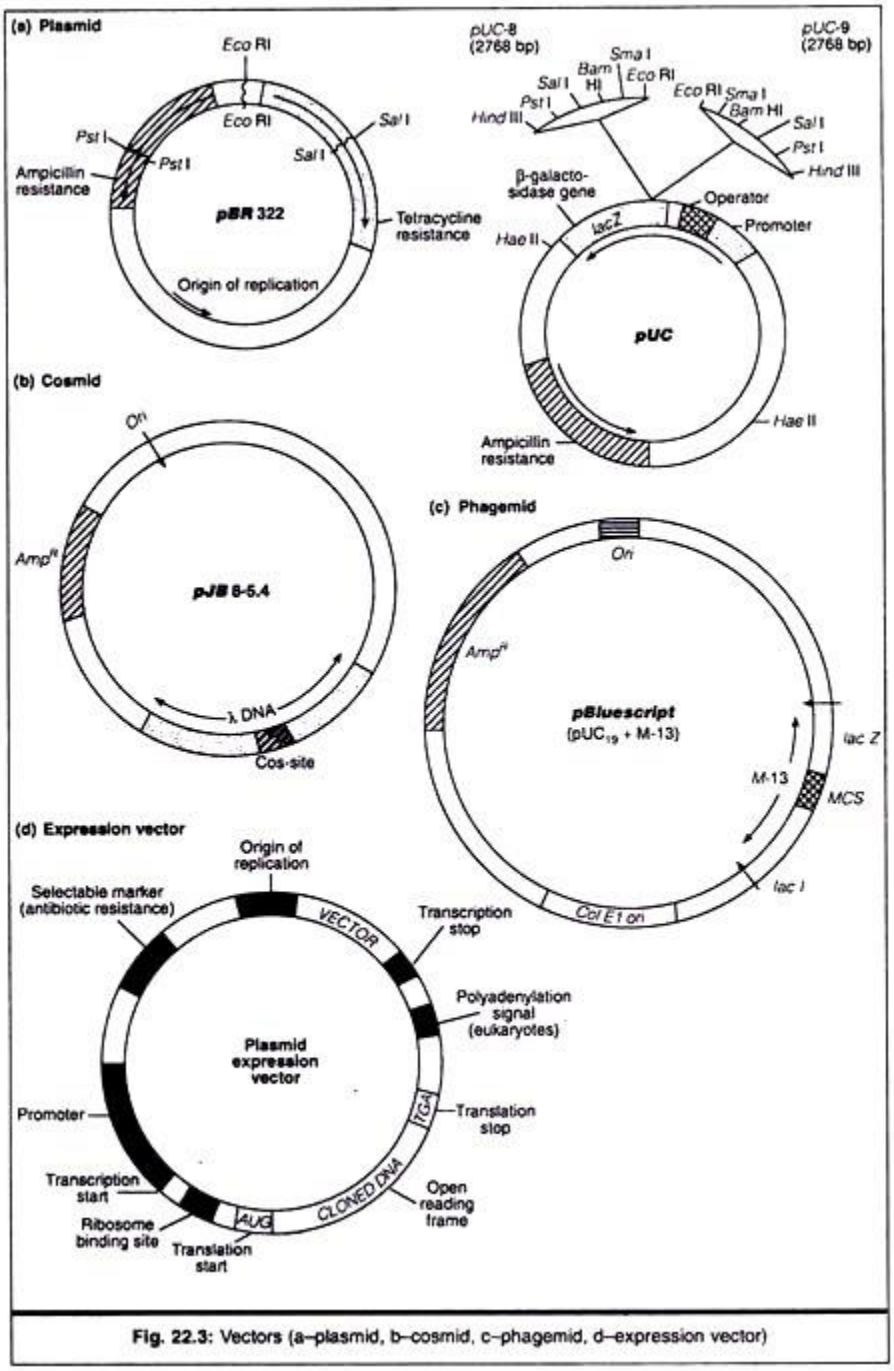


Fig. 22.3: Vectors (a–plasmid, b–cosmid, c–phagemid, d–expression vector)

(a) Plasmid:

Plasmids are the extra-chromosomal, self-replicating, and double stranded closed and circular DNA molecules present in the bacterial cell. A number of properties are specified by plasmids such as antibiotic and heavy metal resistance, nitrogen fixation, pollutant degradation, bacteriocin and toxin production, colicin factors, etc.

Plasmids have following advantages as cloning vehicle (Cohen et a. 1973):

1. It can be readily isolated from the cells.

2. It possesses a single restriction site for one or more restriction enzymes.

3. Insertion of foreign DNA does not alter the replication properties.

4. It can be reintroduced into cell.

5. Selective marker is present.

6. Transformants can be selected easily by using selective medium.:

7. Multiple copy numbers are present in a cell.

Some plasmid vectors are pBR 322, pBR 327, pUC vectors, yeast plasmid vector and Ti, Ri plasmids. Ti and Ri Plasmids are widely used in plant system for genetic transformation.

Among higher plants, Ti plasmid of Agrobacterium tumefaciens or Ri plasmid of A. rhizogenes are the best known vectors. T-DNA, from Ti or Ri plasmid of Agrobacterium, is considered to be very potential for foreign gene transfer in cloning experiments with higher plants.

pBR 322 and pUC Vectors:

pBR322 is a derived plasmid from a naturally occurring plasmid col El, composed of 4362 bp DNA and its replication may be more faster. The plasmid has a point of origin of replication (ori), two selectable marker genes conferring resistance to antibiotics, e.g., ampicillin (amp^r), tetracycline (tet^r) and unique recognition sites for 20 restriction endonucleases.

Tetracycline resistance gene has a cloning site and insertion of foreign segment of DNA will inactivate the tet^r gene. The recombinant plasmid will allow the cells to grow only in presence of ampicillin but will not protect them against tetracycline .

Another plasmid used in gene cloning is pUC vector available in pairs with reverse orders of restriction sites relative to lac^z promoter. This is a synthesized plasmid possessing ampicillin resistance gene (amp^r), origin of replication from pBR322(on) and lac^z J gene from E. coli. pUC 8 and pUC 9 make one such pair.

(b) Bacteriophage:

The bacteriophage has linear DNA molecule, a single break will generate two fragments, foreign DNA can be inserted to generate chimeric phage particle. But as the capacity of phage head is limited, some segments of phage DNA, not having essential genes, may be removed. This technique has been followed in λ (Lambda) phage vectors to clone large foreign particle.

Plasmid can clone up to 20 to 25 kb long fragments of eukaryotic genome. The examples of different Lambda phage vectors are λ gt 10, λ gt 11, EMBL 3, etc. M-13 is a filamentous bacteriophage of E. coli whose single stranded circular DNA has been modified variously to give rise M-13 series of cloning vectors.

(c) Cosmid:

Cosmids are plasmid particles, into which certain specific DNA sequences, namely those for cos sites are inserted which enable the DNA to get packed in X particle. Like plasmids, the cosmids perpetuate in bacteria without lytic development. The cosmids have high efficiency to produce a complete genomic library

(d) Phagemid:

These are prepared artificially by combining features of phages with plasmids. One commonly used phagemid is pBluescript IIKs derived from pUC-19.

(e) Plant and Animal Viruses:

A number of plant and animal viruses have also been used as vectors both for introducing foreign genes into cells and for gene amplification. Cauliflower Mosaic Viruses (CaMV), Tobacco Mosaic Viruses (TMV) and Gemini Virus are three groups of viruses that have been used as vectors for

cloning of DNA segments in plant system. SV 40 (Simian Virus 40), human adenoviruses and retroviruses are potential as vectors for gene transfer into animal cells.

(f) Artifcial Chromosomes:
Yeast Artificial Chromosome (YAC) or Bacterial Artificial Chromosome (BAC) vectors allow cloning of several hundred kb pairs which may represent the whole chromosome. It can be cloned in yeast or bacteria by ligating them to vector sequences that allow their propagation as linear artificial chromosome.

(g) Transposons:
Transposable elements like Ac-Ds or Mu-1 of Maize, P-element of Drosophila may also be used for cloning vector and transfer of gene among eukaryotes.

Expression Vector:
A vector that has been constructed in such a way that inserted DNA molecule is put under appropriate promoter and terminator sequences for high level expression through efficient transcription and translation. Example: Use of promoters ('nos' from T-DNA) or expression cassettes (pRT plasmids) .

Shuttle Vector:
There are plasmids capable of propagating and transferring genes between two organisms (e.g., E. coli and A. tumefciciens). It has unique origins of replication for each cell type as well as different selectable markers. It can, therefore, be used to shuttle gene from prokaryotes to eukaryotes. Example: pBin19

YAC: Yeast Artificial Chromosome

It is based on yeast chromosome system and has following components

- Centromeric sequence: CEN4
- Telomeric sequence: TEL
- Selectable markers: TRP1, URA3, and SUP4.
- Unique restriction site: BamH1 and SnaBI

- Origin of replication

The AAC can be used to clone a DNA fragment up to 600 kb.

In the above vector, the sequence between the BamHI sites is removed. This would yield a linear chromosome which has two arms, Left and Right. The Left arm has TRP1 gene and the Right arm has URA3 as the selectable markers. The yeast used in the cloning experiment needs to be an auxotrophic mutant. The gene TRP1 and URA3 codes for Tryptophan synthase and orotidine 5' phosphate decarboxylase enzyme respectively which are absent in the mutant strain.

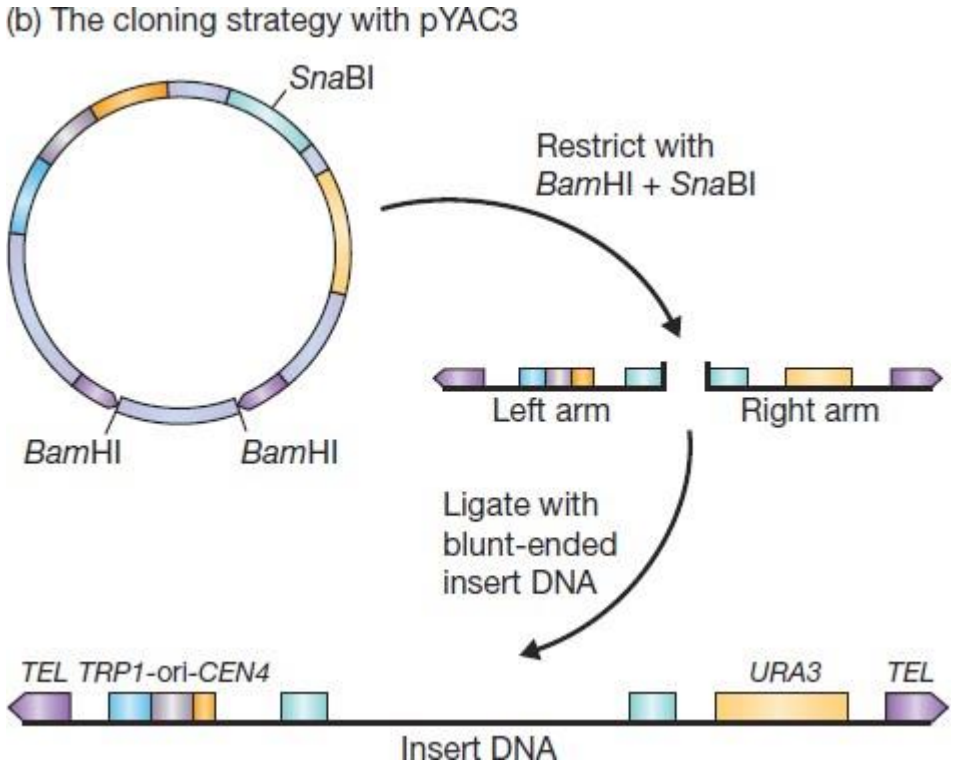


Image adapted from: Brown, T.A., 2016. *Gene cloning and DNA analysis: an introduction*. John Wiley & Sons.

Why we choose two auxotrophic mutant genes?

The answer is to ensure that during the gene ligation, two left arms or two right arms do not ligate with each other. As both of them has been cleaved with BamHI, they can be ligated easily. If this happens then the colonies of yeast won't grow as the cells would lack any one of the above essential genes for the survival in minimal media.

There is a restriction site for SnaBI between SUP4 gene. Here we are ligating the gene of interest. If the gene of interest has been ligated then white colonies are formed. If the vector (Left-Right arm) has been ligated without the gene of interest, then red colonies are formed.

Hence, we can select the recombinant and non-recombinant colonies of the yeast.

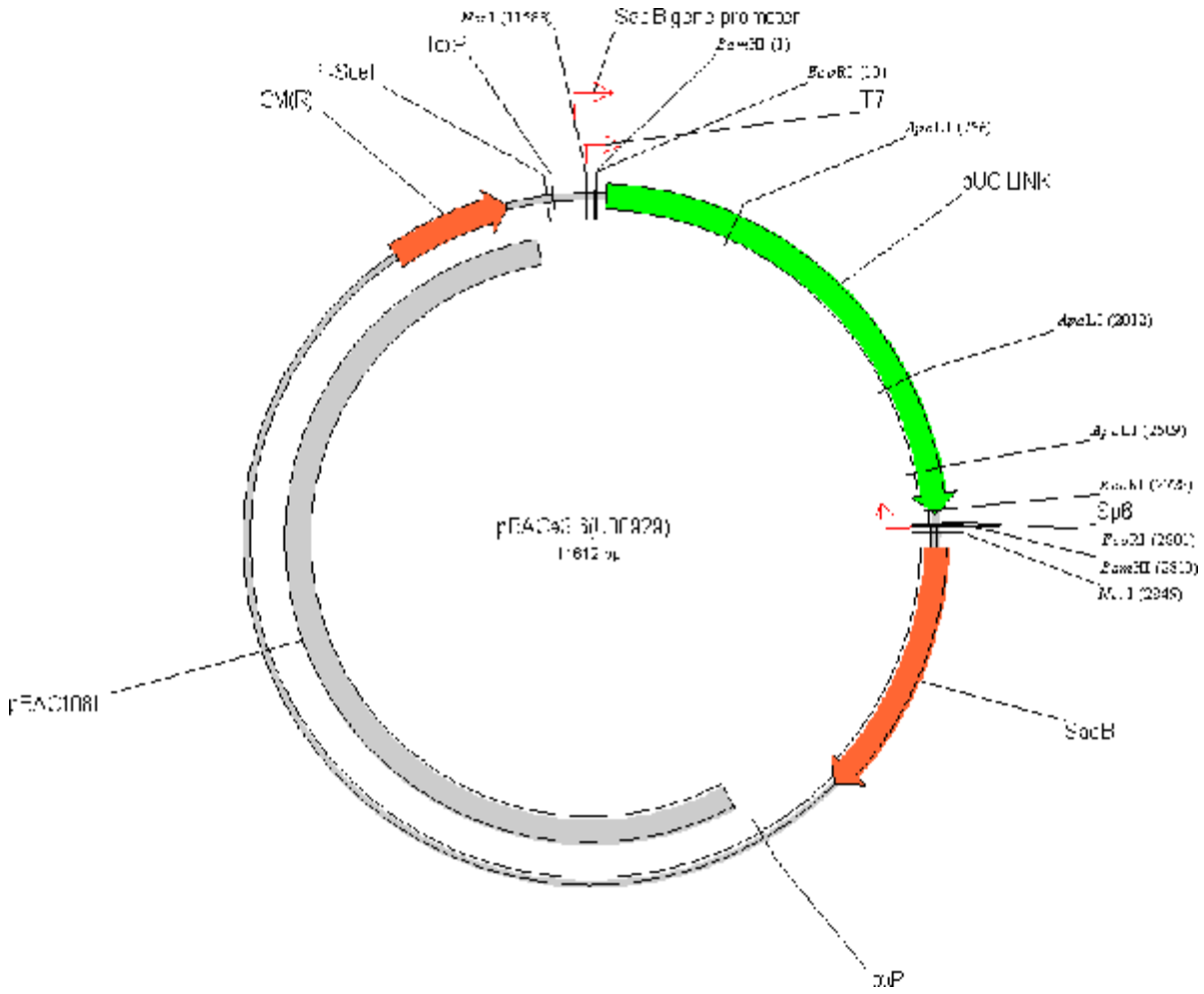
BAC: Bacterial Artificial Chromosomes

It is based on F-plasmid and can clone up to 350 kb of DNA.

The following components are present in the BAC:

- repE: Replication origin- Rolling circle replication.
- PAR-A and PAR-B: Partitioning gene.
- Selectable markers: LacZ' and SacB or Antibiotic resistant gene.
- Promoters: Phage-based promoters- T7 and SP6 promoters.

PAR-A and PAR-B are required for the partitioning of the DNA between the daughter cells. The chromosome is quite bigger in size, which makes it difficult to distribute equally among the daughter cells. Hence, PAR-A and PAR-B derived from the fertility plasmid would facilitate the equal distribution of the DNA.



Selectable markers used are LacZ' and SacB. The transformed, non-recombinant cells would give blue colored colonies in the media having X-gal and IPTG due presence of LacZ' gene. The recombinant cells would form white clones.

If sacB is the selectable marker, then only recombinant cell would grow in the sucrose media. This is because SacB gene synthesis enzyme called **levansucrase** which converts the sucrose into Levan sucrose, a toxic for the cell. Hence, insertion of DNA (recombinant) in between the SacB gene would lead to insertional inactivation of that gene. Thus, no toxic is formed and only recombinant cells grow. The transformed and non-transformed cells need to be screened by other selectable marker.

The selectable marker can also be any antibiotic resistance genes such as chloramphenicol, tetracycline, and ampicillin resistant gene. Different BACs have different types of selectable markers.

Techniques of gene transfer :

The six methods are: (1) Transformation (2) Conjugation (3) Electroporation (4) Liposome-Mediated Gene Transfer (5) Transduction and (6) Direct Transfer of DNA.

Method # 1. Transformation:

Transformation is the method of introducing foreign DNA into bacterial cells (e.g. E.coli). The uptake of plasmid DNA by E.coli is carried out in ice-cold $CaCl_2$ (0-5°C), and a subsequent heat shock (37-45°C for about 90 sec). By this technique, the transformation frequency, which refers to the fraction of cell population that can be transferred, is reasonably good e.g. approximately one cell for 1000 (10^{-3}) cells.

Transformation efficiency:

It refers to the number of trans-formants per microgram of added DNA. For E.coli, transformation by plasmid, the transformation efficiency is about 10^7 to 10^8 cells per microgram of intact plasmid DNA. The bacterial cells that can take up DNA are considered as competent. The competence can be enhanced by altering growth conditions.

The mechanism of the transformation process is not fully understood. It is believed that the $CaCI_2$ affects the cell wall, breaks at localized regions, and is also responsible for binding of DNA to cell surface. A brief heat shock (i.e. the sudden increase in temperature from 5°C to 40°C) stimulates DNA uptake. In general, large-sized DNAs are less efficient in transforming.

Other chemical methods for transformation:

Calcium phosphate (in place of $CaCI_2$) is preferred for the transfer of DNA into cultured cells. Sometimes, calcium phosphate may result in precipitate and toxicity to the cells. Some workers use diethyl amino ethyl dextran (DEAE -dextran) for DNA transfer.

Method # 2. Conjugation:

Conjugation is a natural microbial recombination process. During conjugation, two live bacteria (a donor and a recipient) come together, join by cytoplasmic bridges and transfer single-stranded DNA (from donor to recipient). Inside the recipient cell, the new DNA may integrate with the chromosome (rather rare) or may remain free (as is the case with plasmids).

Conjugation can occur among the cells from different genera of bacteria (e.g Salmonella and Shigella cells). This is in contrast to transformation which takes place among the cells of a bacterial genus. Thus by

conjugation, transfer of genes from two different and unrelated bacteria is possible.

The natural phenomenon of conjugation is exploited for gene transfer. This is achieved by transferring plasmid-insert DNA from one cell to another. In general, the plasmids lack conjugative functions and therefore, they are not as such capable of transferring DNA to the recipient cells. However, some plasmids with conjugative properties can be prepared and used.

Method # 3. Electroporation:

Electroporation is based on the principle that high voltage electric pulses can induce cell plasma membranes to fuse. Thus, electroporation is a technique involving electric field-mediated membrane permeabilization. Electric shocks can also induce cellular uptake of exogenous DNA (believed to be via the pores formed by electric pulses) from the suspending solution.

Electroporation is a simple and rapid technique for introducing genes into the cells from various organisms (microorganisms, plants and animals).

The basic technique of electroporation for transferring genes into mammalian cells is depicted in Fig. 6.11. The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.

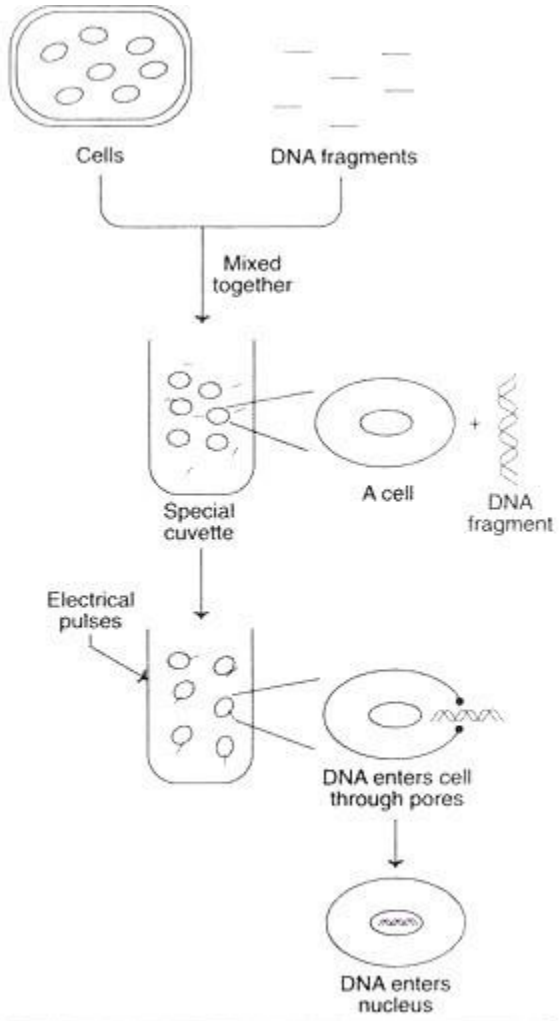


Fig. 6.11 : Gene transfer by electroporation.
(Note : Magnification depicted on right side)

Electroporation is an effective way to transform E.coli cells containing plasmids with insert DNAs longer than 100 kb. The transformation efficiency is around 10^9 transformants per microgram of DNA for small plasmids (about 3kb) and about 10^6 for large plasmids (about 130 kb).

Method # 4. Liposome-Mediated Gene Transfer:

Liposomes are circular lipid molecules, which have an aqueous interior that can carry nucleic acids. Several techniques have been developed to encapsulate DNA in liposomes. The liposome- mediated gene transfer, referred to as lipofection, is depicted in Fig. 6.12.

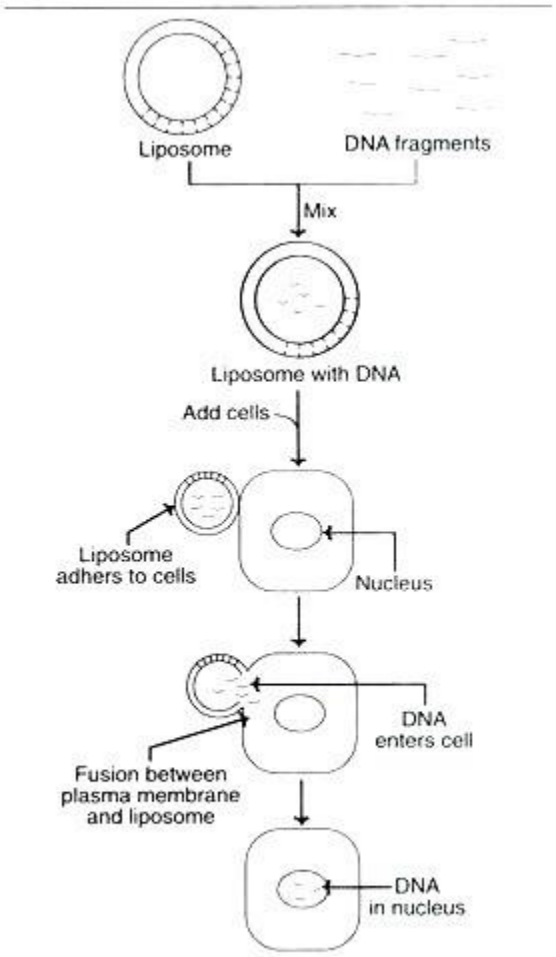


Fig. 6.12 : Liposome-mediated gene transfer
(Note : For clarity, the native cell DNA is not shown).

On treatment of DNA fragment with liposomes, the DNA pieces get encapsulated inside liposomes. These liposomes can adher to cell membranes and fuse with them to transfer DNA fragments. Thus, the DNA enters the cell and then to the nucleus. The positively charged liposomes very efficiently complex with DNA, bind to cells and transfer DNA rapidly.

Lipofection is a very efficient technique and is used for the transfer of genes to bacterial, animal and plant cells. T

Method # 5. Transduction:

Sometimes, the foreign DNA can be packed inside animal viruses. These viruses can naturally infect the cells and introduce the DNA into host cells. The transfer of DNA by this approach is referred to as transduction.

Method # 6. Direct Transfer of DNA:

It is possible to directly transfer the DNA into the cell nucleus. Microinjection and particle bombardment are the two techniques commonly used for this purpose.

Microinjection:

DNA transfer by microinjection is generally used for the cultured cells. This technique is also useful to introduce DNA into large cells such as oocytes, eggs and the cells of early embryos. The term transfection is used for the transfer DNA into eukaryotic cells, by various physical or chemical means.

PCR :

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. This tool is commonly used in the molecular biology and biotechnology labs.

Principle of PCR :

The principle of PCR is based on the enzymatic replication of nucleic acids. PCR involves the use of primer mediated enzymes for the amplification of DNA. DNA Polymerase synthesises 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.

PCR Steps

The PCR involves three major cyclic reactions:

Denaturation

Denaturation occurs when the reaction mixture is heated to 94℃ for about 15-30 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.

Annealing

The reaction temperature is lowered to 54-60℃ for around 20-40 seconds. Here, the primers bind to their complementary sequences on the template DNA.

Primers are single strand sequences of DNA or RNA around 20 to 30 bases in length.

They serve as the starting point for the synthesis of DNA.

The two separated strands run in the opposite direction and consequently there are two primers- a forward primer and a reverse primer.

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.

Methods of DNA sequencing :

DNA sequencing is the process of determining the nucleic acid sequence – the order of nucleotides in DNA. It includes any method or technology that is used to determine the order of the four bases: adenine, guanine, cytosine, and thymine. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery.[1][2]

Knowledge of **DNA sequences** has become indispensable for basic biological research, and in numerous applied fields such as medical diagnosis, biotechnology, forensic biology, virology and biological systematics. Comparing healthy and mutated DNA sequences can diagnose different diseases including various cancers,[3] characterize antibody repertoire,[4] and can be used to guide patient treatment.[5] Having a quick way to sequence DNA allows for faster and more individualized medical care to be administered, and for more organisms to be identified and cataloged.[4]

The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes, of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species.

The first DNA sequences were obtained in the early 1970s by academic researchers using laborious methods based on two-dimensional chromatography. Following the development of fluorescence-based sequencing methods with a DNA sequencer, DNA sequencing has become easier and orders of magnitude faster.

Applications :

DNA sequencing may be used to determine the sequence of individual genes, larger genetic regions (i.e. clusters of genes or operons), full chromosomes, or entire genomes of any organism. DNA sequencing is also the most efficient way to indirectly sequence RNA or proteins (via their open reading frames). In fact, DNA sequencing has become a key technology in many areas of biology and other sciences such as medicine, forensics, and anthropology.

Molecular biology

Sequencing is used in molecular biology to study genomes and the proteins they encode. Information obtained using sequencing allows researchers to identify changes in genes, associations with diseases and phenotypes, and identify potential drug targets.

Evolutionary biology

Since DNA is an informative macromolecule in terms of transmission from one generation to another, DNA sequencing is used in evolutionary biology to study how different organisms are related and how they evolved.

Metagenomics

The field of metagenomics involves identification of organisms present in a body of water, sewage, dirt, debris filtered from the air, or swab samples from organisms. Knowing which organisms are present in a particular environment is critical to research in ecology, epidemiology, microbiology, and other fields. Sequencing enables researchers to determine which types of microbes may be present in a microbiome, for example.

Virology

Virology

As most viruses are too small to be seen by a light microscope, sequencing is one of the main tools in virology to identify and study the virus. Traditional Sanger sequencing and next-generation sequencing are used to sequence viruses in basic and clinical research, as wells as for the diagnosis of emerging viral infections, molecular epidemiology of viral pathogens, and drug-resistance testing. There are more than 2.3 million unique viral sequences in GenBank . Recently, NGS has surpassed traditional Sanger as the most popular approach for generating viral genomes.

Medicine

Medical technicians may sequence genes (or, theoretically, full genomes) from patients to determine if there is risk of genetic diseases. This is a form of genetic testing, though some genetic tests may not involve DNA sequencing. Also, DNA sequencing may be useful for determining a specific bacteria, to allow for more precise antibiotics treatments, hereby reducing the risk of creating antimicrobial resistance in bacteria populations.

Forensics

DNA sequencing may be used along with DNA profiling methods for forensic identification and paternity testing. DNA testing has evolved tremendously in the last

few decades to ultimately link a DNA print to what is under investigation. The DNA patterns in fingerprint, saliva, hair follicles, etc. uniquely separate each living organism from another. Testing DNA is a technique which can detect specific genomes in a DNA strand to produce a unique and individualized pattern.

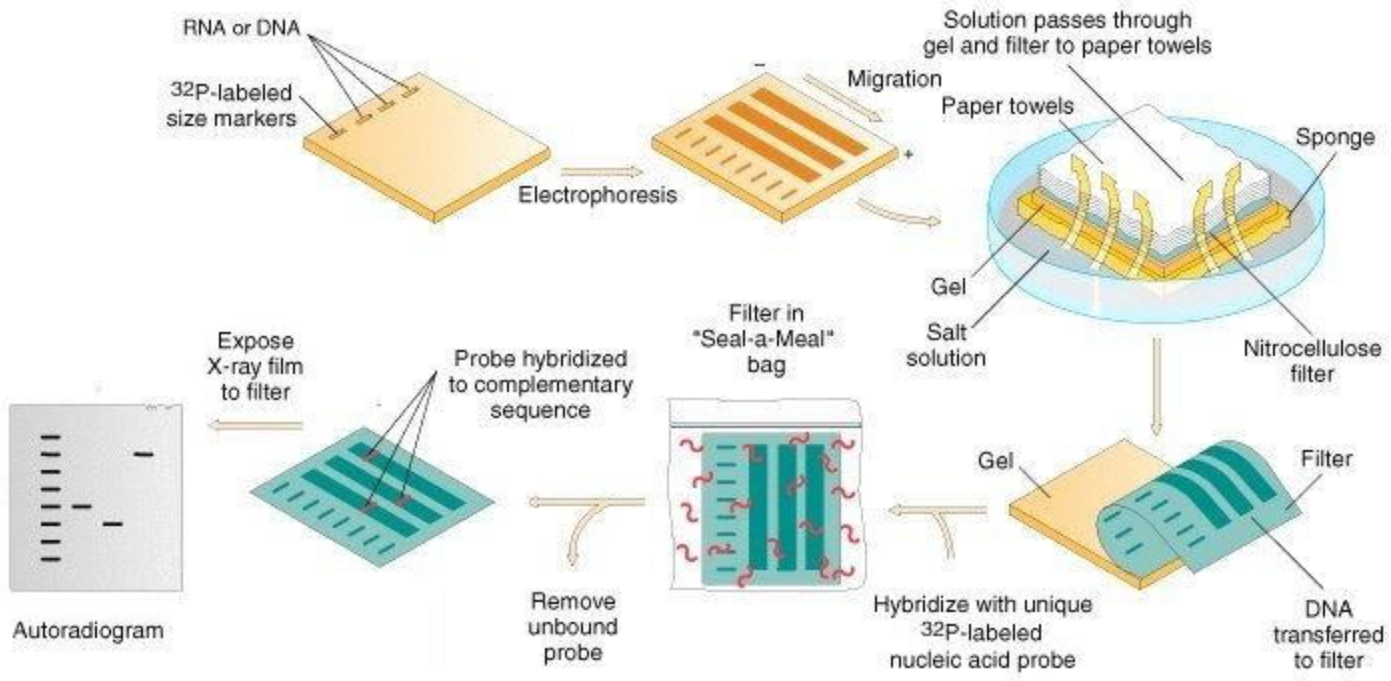
• Southern Blot

- The analytical technique that involves the transfer of a specific DNA, RNA or a protein separated on gel to a carrier membrane, for their detection or identification is termed as blotting.
- The process of transfer of the denatured fragments out of the gel and onto a carrier membrane makes it accessible for analysis using a probe or antibody.
- Depending upon the substance to be separated, blotting techniques may be – Southern blot, Northern blot or Western blot which separates DNA, RNA and proteins respectively.
- **Southern Blot** is the analytical technique used in molecular biology, immunogenetics and other molecular methods to detect or identify DNA of interest from a mixture of DNA sample or a specific base sequence within a strand of DNA.
- The technique was developed by a molecular biologist E.M. Southern in 1975 for analysing the related genes in a DNA restriction fragment and thus named as Southern blotting in his honour.

Principle of Southern Blot

The process involves the transfer of electrophoresis-separated DNA fragments to a carrier membrane which is usually nitrocellulose and the subsequent detection of the target DNA fragment by probe hybridization. Hybridization refers to the process of forming a double-stranded DNA molecule between a single-stranded DNA probe and a single-stranded target DNA. Since the probe and target DNA are complementary to each other, the reaction is specific which aids in the detection of the specific DNA fragment.

Steps Involved in Southern Blot



1. **Extraction and purification of DNA from cells**

DNA is first separated from target cells following standard methods of genomic DNA extraction and then purified.

2. **Restriction Digestion or DNA Fragmentation**

Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments. One or more restriction enzymes can be used to achieve such fragments.

3. **Separation by Electrophoresis**

The separation may be done by agarose gel electrophoresis in which the negatively charged DNA fragments move towards the positively charged anode, the distance moved depending upon its size.

4. **Depurination**

Partial depurination is done by the use of dilute HCl which promotes higher efficiency transfer of DNA fragments by it breaking down into smaller pieces.

5. **Denaturation**

DNA is then denatured with a mild alkali such as an alkaline solution of NaOH. This causes the double stranded DNA to become single-stranded, making them suitable for hybridization. DNA is then neutralized with NaCl to prevent re-hybridization before addition of the probe.

6. **Blotting**

The denatured fragments are then transferred onto a nylon or nitrocellulose filter membrane which is done by placing the gel on top of a buffer saturated

filter paper, then laying nitrocellulose filter membrane on the top of gel. Finally some dry filter papers are placed on top of the membrane. Fragments are pulled towards the nitrocellulose filter membrane by capillary action and result in the contact print of the gel.

7. Baking

The nitrocellulose membrane is removed from the blotting stack, and the membrane with single stranded DNA bands attached on to it is baked in a vacuum or regular oven at 80 °C for 2-3 hours or exposed to ultraviolet radiation to permanently attach the transferred DNA onto the membrane.

8. Hybridization

The membrane is then exposed to a hybridization probe which is a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labeled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye.

9. Washing of unbound probes

After hybridization, the membrane is thoroughly washed with a buffer to remove the probe that is bound nonspecifically or any unbound probes present.

10. Autoradiograph

The hybridized regions are detected autoradiographically by placing the nitrocellulose membrane in contact with a photographic film which shows the hybridized DNA molecules. The pattern of hybridization is visualized on X-ray film by autoradiography in case of a radioactive or fluorescent probe is used or by the development of color on the membrane if a chromogenic detection method is used.

Applications of Southern Blot

- Identifying specific DNA in a DNA sample.
- Preparation of RFLP (Restriction Fragment Length Polymorphism) maps
- Detection of mutations, deletions or gene rearrangements in DNA
- For criminal identification and DNA fingerprinting (VNTR)
- Detection and identification of trans gene in transgenic individual
- Mapping of restriction sites
- For diagnosis of infectious diseases
- Prognosis of cancer and prenatal diagnosis of genetic diseases
- Determination of the molecular weight of a restriction fragment and to measure relative amounts in different samples.

Genomic Libraries:

- **Meaning of Genomic Libraries:**
- Genomic libraries are libraries of genomic DNA sequences. These can be produced using DNA from any organism.
- **cDNA Library:**

- **Meaning of cDNA Library:**
- A cDNA library is defined as a collection of cDNA fragments, each of which has been cloned into a separate vector molecule.
- **Principle of cDNA Library:**
- In the case of cDNA libraries we produce DNA copies of the RNA sequences (usually the mRNA) of an organism and clone them. It is called a cDNA library because all the DNA in this library is complementary to mRNA and are produced by the reverse transcription of the latter.
- Much of eukaryotic DNA consists of repetitive sequences that are not transcribed into mRNA and the sequences are not represented in a cDNA library. It must be noted that prokaryotes and lower eukaryotes do not contain introns, and preparation of cDNA is generally unnecessary for these organisms. Hence, cDNA libraries are produced only from higher eukaryotes.
- **Vectors used in the Construction of cDNA Library:**
- Both the bacterial and bacteriophage DNA are used as vectors in the construction of cDNA library.
- **The following table give a detailed information:**

Table 6.2: Vectors used in the construction of cDNA library

Vectors	Insert size	Remarks
λ-phages	Up to 20-30kb (for replacement vectors) and 10-15kb (for insertion vectors)	• Maximum size of mRNA is about 8kb. Hence the capicity of DNA insert is not a major conern • Insertion vector system is usually employed • Useful for study of individual genes and their putative functions • Efficient packaging system, easy for gene transfer into *E. coli*, more representative than plasmid libraries, subcloning and subsequent DNA manipulation process are less convenient than plasmid systems
Bacterial plasmids	Up to 10-15kb	• Relatively easy to transform *E. coli* cells although may not be efficient as the λ-phage system for large scale gene transfer • Less representative than λ-phage libraries, subcloning and subsequent DNA manipulation processes are more convenient than the λ-pahge systems.

- **Procedure in the Construction of cDNA Library:**
- **The steps involved in the construction of a cDNA library are as follows:**
- **1. Extraction of mRNA from the eukaryotic Cell:**
- Firstly, the mRNA is obtained and purified from the rest of the RNAs. Several methods exist for purifying RNA such as trizol extraction and column purification. Column purification is done by using oligomeric dT nucleotide coated resins where only the mRNA having the poly-A tail will bind.
- The rest of the RNAs are eluted out. The mRNA is eluted by using eluting buffer and some heat to separate the mRNA strands from oligo-dT.
- **2. Construction of cDNA from the Extracted mRNA (Fig. 6.4):**
- **There are different strategies for the construction of a cDNA. These are discussed as follows:**

- **(a) The RNase Method:**
- The principle of this method is that a complementary DNA strand is synthesized using reverse transcriptase to make an RNA: DNA duplex. The RNA strand is then nicked and replaced by DNA. In this method the first step is to anneal a chemically synthesized oligo-dT primer to the 3′ polyA-tail of the RNA.
- The primer is typically 10-15 residues long, and it primes (by providing a free 3′ end) the synthesis of the first DNA strand in the presence of reverse transcriptase and deoxyribonucleotides. This leaves an RNA: DNA duplex.
- The next step is to replace the RNA strand with a DNA strand. This is done by using RNase H enzyme which removes the RNA from RNA: DNA duplex. The DNA strand thus left behind is then considered as the template and the second DNA strand is synthesized by the action of DNA polymerase II.
- **(b) The Self-Priming method:**
- This involved the use of an oligo-dT primer annealing at the polyadenylate tail of the mRNA to prime first DNA strand synthesis against the mRNA. This cDNA thus formed has the tendency to transiently fold back on itself, forming a hairpin loop. This results in the self-priming of the second strand.
- After the synthesis of the second DNA strand, this loop must be cleaved with a single-strand-specific nuclease, e.g., SI nuclease, to allow insertion into the cloning vector. This method has a serious disadvantage. The cleavage with SI nuclease results in the loss of a certain amount of sequence at the 5′ end of the clone.

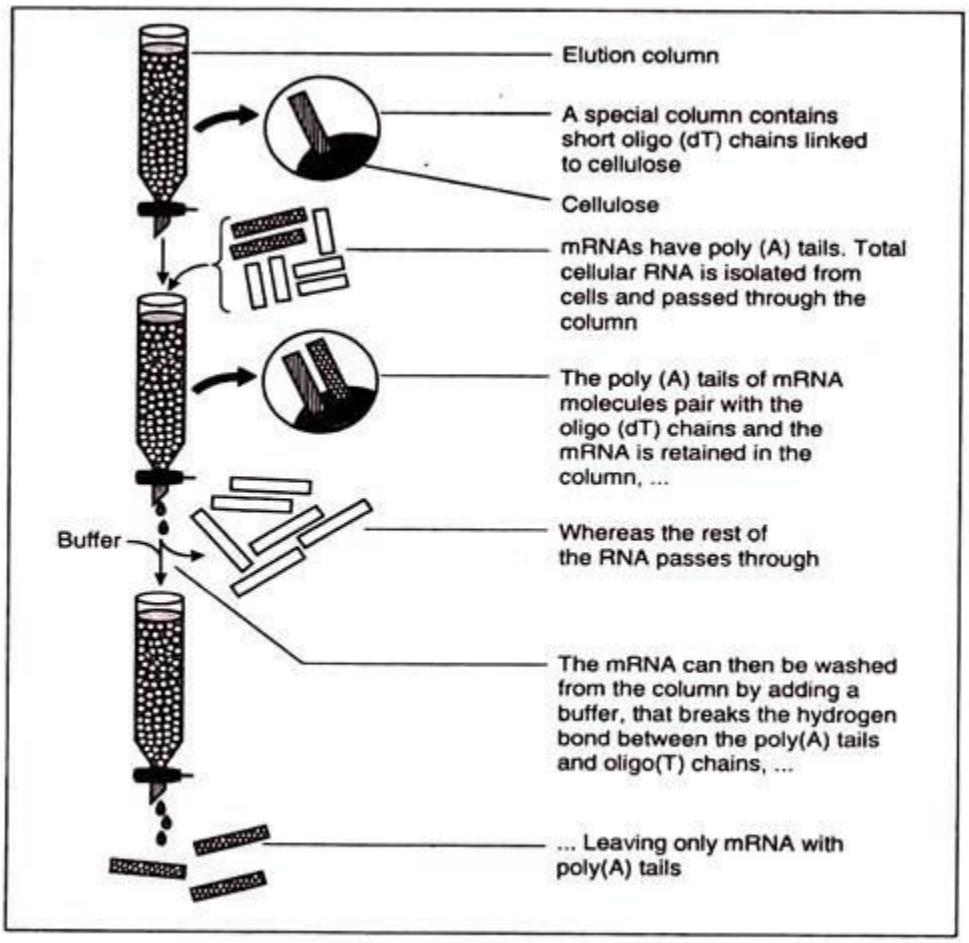


Fig. 6.4: Extraction of mRNA from the eukaryotic cell

- **(c) Land et al. Strategy:**
- After first-strand synthesis, which is primed with an oligo- dT primer as usual, the cDNA is tailed with a string of cytidine residues using the enzyme terminal transferase. This artificial oligo-dC tail is then used as an annealing site for a synthetic oligo-dG primer, allowing synthesis of the second strand.
- **(d) Homopolymer Tailing:**
- This approach uses the enzyme terminal transferase, which can polymerize nucleotides onto the 3′-hydroxyl of both DNA and RNA molecules. We carry out the synthesis of the first DNA strand essentially as before, to produce an RNA: DNA hybrid.
- We then use terminal transferase and a single deoxyribonucleotide to add tails of that nucleotide to the 3′ ends of both RNA and DNA strands. The result of this is that the DNA strand now has a known sequence at its 3′ end Typically, dCTP or dATP are used.

- A complementary oligomer (synthesized chemically) can now be annealed and used as a primer to direct second strand synthesis. This oligomer (and also the one used for first strand synthesis) may additionally incorporate a restriction site, to help in cloning the resulting double- stranded cDNA.

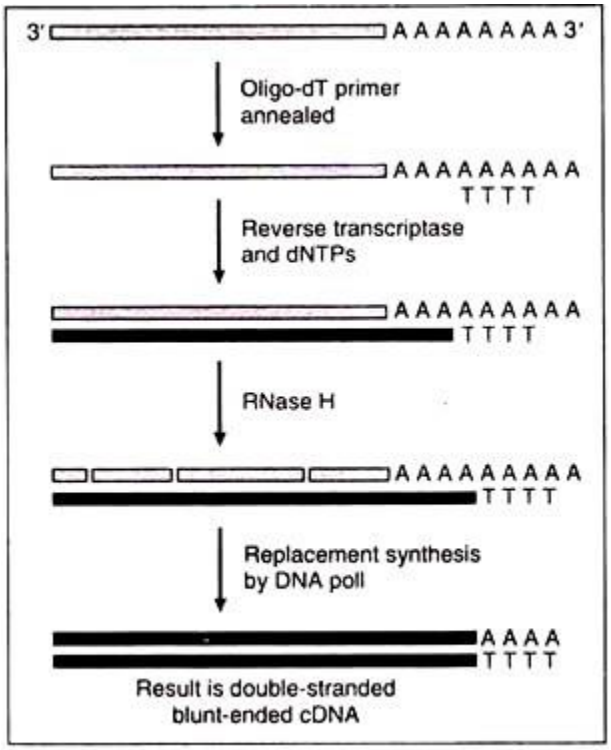


Fig. 6.5: The RNase H method of c-DNA synthesis

- **(e) Rapid Amplifcation of cDNA Ends (RACE):**
- It is sometimes the case that we wish to clone a particular cDNA for which we already have some sequence data, but with particular emphasis on the integrity of the 5′ or 3′ ends. RACE techniques (Rapid Amplification of cDNA Ends) are available for this. The RACE methods are divided into 3'RACE and 5'RACE, according to which end of the cDNA we are interested in.

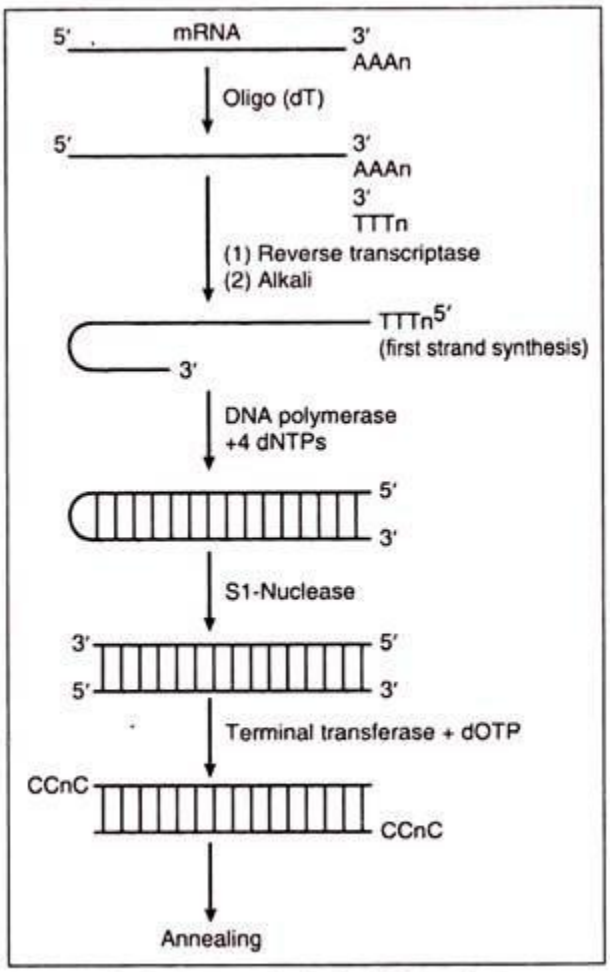


Fig. 6.6: Self-priming method of c-DNA synthesis

- **(a) 3'RACE:**
- In this type of RACE, reverse transcriptase synthesis of a first DNA strand is carried out using a modified oligo-dT primer. This primer comprises a stretch of unique adaptor sequence followed by an oligo-dT stretch. The first strand synthesis is followed by a second strand synthesis using a primer internal to the coding sequence of interest.
- **This is followed by PCR using**
- (i) The same internal primer and '
- (ii) The adaptor sequence (i.e., omitting the oligo-dT). Although in theory it should be possible to use a simple oligo- dT primer throughout instead of the adaptor-oligo-dT and adaptor combination, the low melting temperature for an oligo-dT primer may interfere with the subsequent rounds of PCR.
- **(b) 5'RACE:**

- In this type of RACE first cDNA strand is synthesized with re- verse transcriptase and a primer from within the coding sequence. Unincorporated primer is removed and the cDNA strands are tailed with oligo-dA. A second cDNA strand is then synthesized with an adaptor-oligo-dT primer.
- **The resulting double-stranded molecules are then subject to PCR using**
- (i) A primer nested within the coding region and
- (ii) The adaptor sequence. A nested primer is used in the final PCR to improve specificity. The adaptor sequence is used in the PCR because of the low melting temperature of a simple oligo-dT primer, as in 3'RACE above. A number of kits for RACE are commercially available.

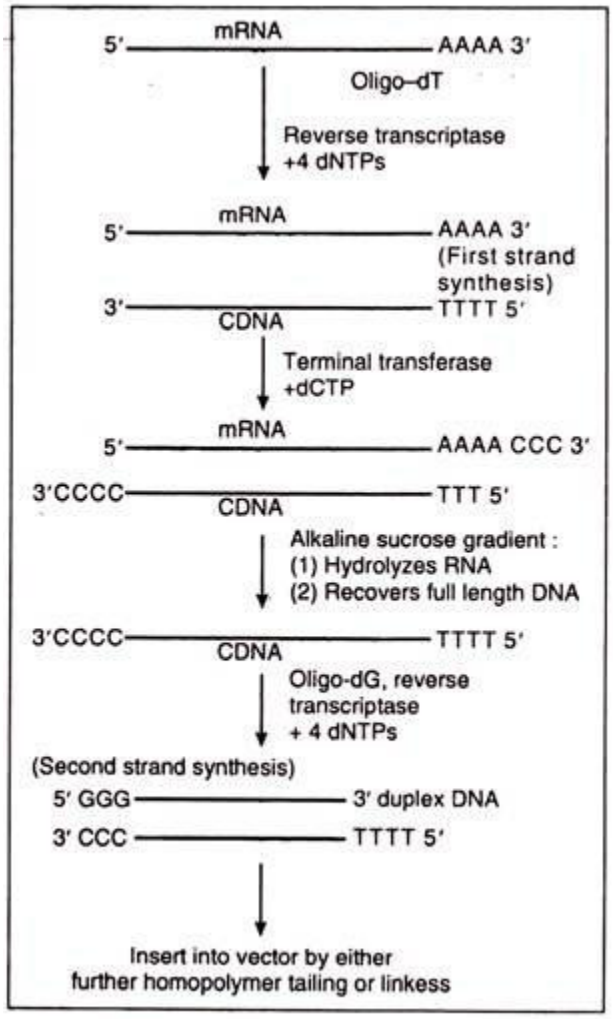


Fig. 6.7: Land et al. strategy

- **3. Cloning the c-DNA:**

- **(a) Linkers:**
- The RNaseH and homopolymer tailing methods ultimately generate a collection of double-stranded, blunt-ended cDNA molecules. They must now be attached to the vector molecules. This could be done by blunt-ended ligation, or by the addition of linkers, digestion with the relevant enzyme and ligation into vector.
- **(b) Incorporation of Restriction Sites:**
- It is possible to adapt the homopolymer tailing method by using primers that are modified to incorporate restriction. In the diagram shown next page, the oligo-dT primer is modified to contain a restriction site (in the figure, a Sail site GTCGAC).
- The 3′ end of the newly synthesized first cDNA strand is tailed with C's. An oligo-dG primer, again preceded by a Sail site within a short double-stranded region of the oligonucleotide, is then used for second-strand synthesis.
- Note that this method requires the use of an oligonucleotide containing a double-stranded region. Such oligonucleotides are made by synthesizing the two strands separately and then allowing them to anneal to one another.

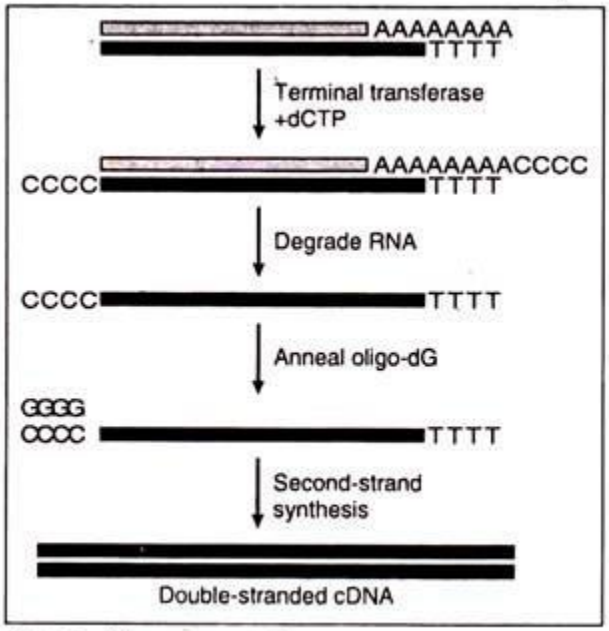


Fig. 6.8: Homopolymer tailing

- **(c) Homopolymer Tailing of cDNA:**

- Another option is to use terminal transferase again. Treatment of the blunt-ended double-stranded cDNA with terminal transferase and dCTP leads to the polymerization of several C residues (typically 20 or so) to the 3′ hydroxyl at each end.
- Treatment of the vector with terminal transferase and dGTP leads to the incorporation of several G residues onto the ends of the vector. (Alternatively, dATP and dTTP can be used.) The vector and cDNA can now anneal, and the base-paired region is often so extensive that treatment with DNA ligase is unnecessary.
- In fact, there may be gaps rather than nicks at the vector insert boundaries, but these are repaired by physiological processes once the recombinant molecules have been introduced into a host.

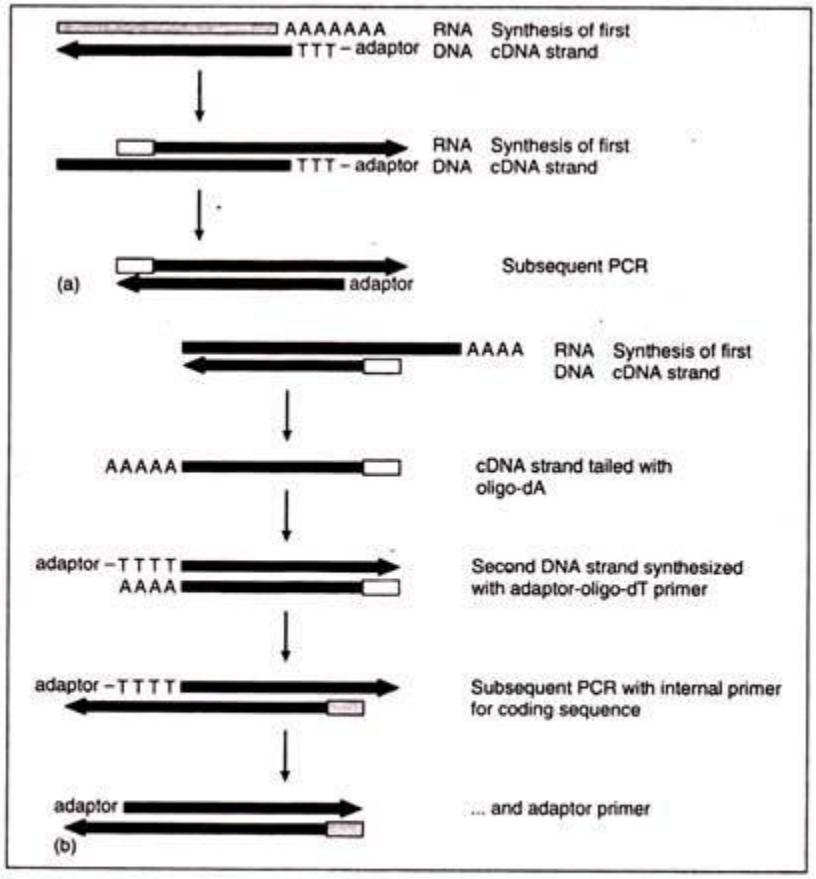


Fig. 6.9: RACE. (a) 3′RACE. The first primer is the oligo-dT-adaptor molecule. The second primer (open box) is internal to the coding sequence of interest. This is used in conjunction with the adaptor primer (rather than the oligo-dT-adaptor primer) in subsequent PCR. (b) 5′RACE. Synthesis of the first cDNA strand uses a primer within the coding region (open box). The first cDNA strand is tailed with oligo-dA. A second DNA strand is synthesized with an adaptor-oligo-dT primer. This is followed by PCR with (i) a primer nested within the coding sequence (shaded box) and (ii) the adaptor.

-

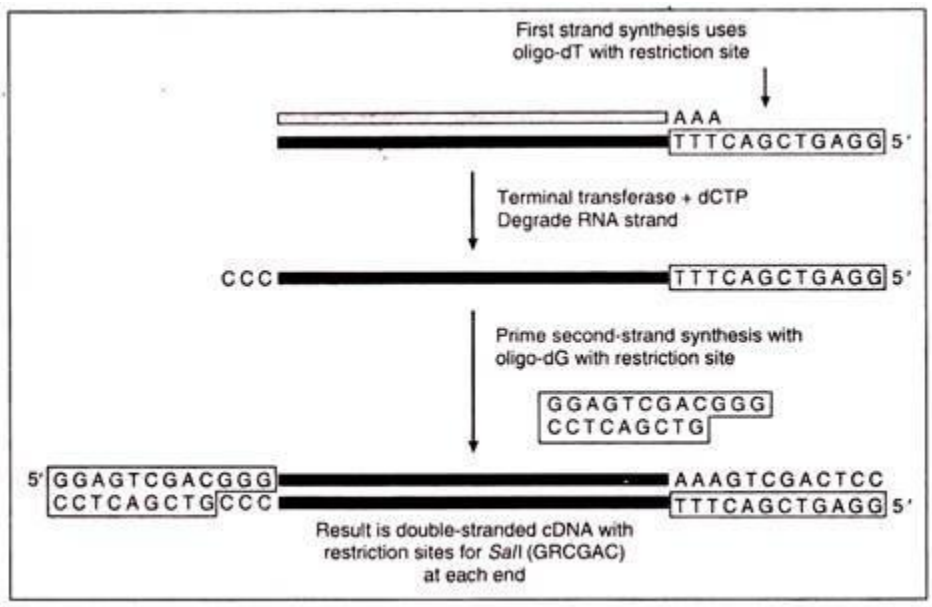


Fig. 6.10: Modification of homopolymer tailing, incorporating restriction sites

- **Advantages of cDNA Library:**
- A cDNA library has two additional advantages. First, it is enriched with fragments from actively transcribed genes. Second, introns do not interrupt the cloned sequences; introns would pose a problem when the goal is to produce a eukaryotic protein in bacteria, because most bacteria have no means of removing the introns.
- **Disadvantages of cDNA Library:**
- The disadvantage of a cDNA library is that it contains only sequences that are present in mature mRNA. Introns and any other sequences that are altered after transcription are not present; sequences, such as promoters and enhancers, that are not transcribed into RNA also are not present in a cDNA library.
- It is also important to note that the cDNA library represents only those gene sequences expressed in the tissue from which the RNA was isolated. Furthermore, the frequency of a particular DNA sequence in a cDNA library depends on the abundance of the corresponding mRNA in the given tissue. In contrast, almost all genes are present at the same frequency in a genomic DNA library.
- **Applications of cDNA Library:**
- **Following are the applications of cDNA libraries:**
- 1. Discovery of novel genes.

- 2. Cloning of full-length cDNA molecules for in vitro study of gene function.
- 3. Study of the repertoire of mRNAs expressed in different cells or tissues.
- 4. Study of alternative splicing in different cells or tissues.

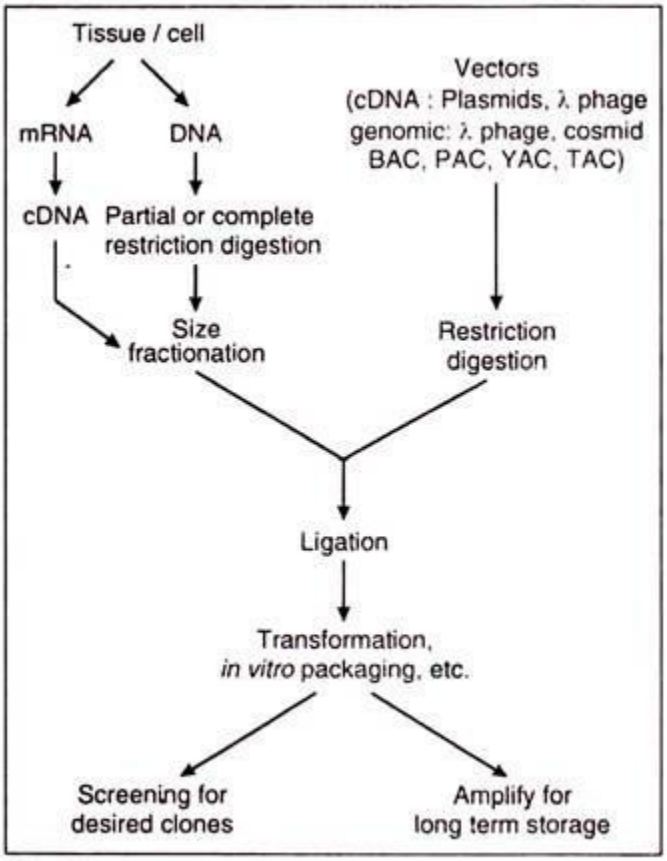


Fig. 6.11: Flow chart showing the construction of genomic and cDNA library

Cell Culture

- Basics, Techniques and Media -

Essentially, **cell culture** involves the distribution of cells in an artificial environment (in vitro) which is composed of the necessary nutrients, ideal temperature, gases, pH and humidity to allow the cells to grow and proliferate.

- **In vivo** - When the study involves living biological entities within the organism.

- **In vitro** - When the study is conducted using biological entities (cells, tissue etc) that has been isolated from their natural biological environment. E.g. tissue or cells isolated from the liver or kidney

- Whereas pieces of tissue can be put in the appropriate culture to produce cells that can then be used for culture (explant culture), cells from tissues (soft tissue) can be obtained through enzymatic reactions. Here, such enzymes as trypsin and proname are used to break down the tissue and release the desired cells.
- When cells have been obtained directly from the organism/animal tissue (or even plant tissue) through enzymatic or mechanical techniques, such cells are referred to as primary cells. However, cells that continue to proliferate indefinitely (after the first subculture) under special conditions are referred to as cell lines.
- These particular cells tend to have been passaged for a long period of time, which causes them to acquire homogenous (similar) genotypic and phenotypical traits.

Morphology

Based on their appearance, cells in culture can be categorized in to three main groups:

- Fibroblastic - This includes cells that tend to be bipolar/multipolar with elongated shapes. These cells are attached to the substrate as they grow.

- Epithelial - Epithelial like cells attain a polygonal shape with regular dimensions. Although they tend to grow in discrete patches, these cells also grow attached to the substrate. There are two major types of culture media. These include: Lymphoblast - These cells are usually spherical in shape and do not attach to the surface of the substrate. As a result, they are grown in suspension.

***** For solid plates, solidifying agents (such as agar) are used where the liquid media is used with agar.

Natural media - Natural culture media is composed of biological fluids that are naturally occurring. Although this type of media can be used for a range of cells, its biggest disadvantage is that it may lack the exact components required by given cells, which can greatly affect reproducibility.

Artificial media - Also referred to as synthetic media, artificial media refers to the type of media that is produced by adding such nutrients as vitamins, gases (oxygen and carbon dioxide) and protein among others. These organic

and inorganic nutrients are added so as to meet the specific needs of given cells, and thus provide the ideal environment for their growth.

As such, they can be used for a number of purposes including:

- Providing immediate survival of the cells
- Allowing for prolonged survival of the cells
- Allowing for indefinite growth of the cells
- Providing for specialized functions

On the other hand, culture may be categorized as:

Selective media- This is a special type of media that only allows for certain cells to grow. For instance, blood agar (used to isolate Streptococcus & Moraxella species) can be turned in to a selective media by adding antibiotics.

Differential media- This type of media allows for different types of cells/microorganisms to grow depending on their metabolism.

As mentioned above, different types of synthetic media are prepared in a manner that will provide the ideal proliferation environment for given cells. For this reason, synthetic media can be divided in to four major categories.

These include:

Serum containing media - In these types of media, serum (fetal bovine serum) is used as a carrier for nutrients and growth factors among others that tend to be water insoluble.

Serum-free media - These types of media is typically produced for the purposes of supporting single cell type of culture. As such, it provides specified nutrients and other factors required by the cell type. In this media, serum is absent because it present some disadvantages and can result in misinterpretation of immunological results.

Chemically defined media - Like the name suggests, this type of media is composed of contamination- free pure organic and inorganic ingredients. Constituents of this type of media are typically produced through genetic engineering in bacteria/yeast.

Protein-free media - Protein- free media are typically lacking of any type of protein. It is largely used to promote superior growth of the cells as well as protein expression in addition to facilitating for the purification of any expressed product.

Some of the major components of cell culture media include:

- Nutrients - provided for by peptides and amino-acids, which are the building blocks of proteins
- Carbohydrates for energy
- Essential minerals such as calcium, magnesium, phosphates and iron among others buffering agents such s acetates to stabilize the culture media
- Vitamins
- PH change indicators such as phenol red

Cell culture media are used for the proliferation of cells, which can then be identified and studied. As such, it can be used for various purposes including for education, diagnosis and treatment of a disease among others.

Cell Suspension :

In culture methods, cell suspension refers to a type of culture where cells are suspended in a liquid medium.

To obtain single cells, a friable callus (small tissue that falls apart easily) is put in agitated liquid medium (agitation allows for gaseous exchange unlike solid medium), breaking it up. This allows for single cells to be released, which are then transferred to another fresh medium.

Cell suspension cultures have a big advantage over the stationary ones given that it allows for the cells to be uniformly bathed. Moreover, given that the medium tends to be agitated, it allows for aeration of the medium, providing gases to the cells. Given that the medium is a suspension, it also becomes easy to manipulate the contents of the culture.

Like any other culture, suspension cell culture has to be under controlled conditions, proving the cells with an ideal environment to proliferate. Once they reach about 80 percent confluence, it is time to subculture in order to ensure continued proper growth.

***** 80 percent confluence refers to the state where 80 percent of the culture surface is covered with the growing cells.

In some cases, the cells in suspension may adhere on to the plastic surface of the culture flask or even form clumps. In such cases, a pipette can be used to pick these cells and expel them on to the surface of the flask and therefore away from the plastic surface. This helps obtain single cells given that they are no adhere on to the plastic surface.

Lymphoblast - These cells are usually spherical in shape and do not attach to the surface of the substrate. As a result, they are grown in suspension.

***** For solid plates, solidifying agents (such as agar) are used where the liquid media is used with agar.

Cell Culture Media :

Cell culture is one of the major techniques in the life sciences. It is the general term used for 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. Cell culture media generally 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.

Types of Cell Culture Media

Animal cells can be cultured either using a completely natural medium or an artificial/synthetic medium along with some natural products.

	Media Type	Examples	Uses
Natural media	Biological Fluids	plasma, serum, lymph, human placental cord serum, amniotic fluid	
	Tissue Extracts	Extract of liver, spleen, tumors, leucocytes and bone marrow, extract of the bovine embryo and chick embryo	
	Clots	coagulants or plasma clots	
Artificial media	Balanced salt solutions	PBS, DPBS, HBSS, EBSS	Form the basis of complex media
	Basal media	MEM DMEM	Primary and diploid culture
	Complex media	RPMI-1640, IMDM	Supports a wide range of mammalian cells

Table 1. Types of natural and artificial media.

Natural media

Natural media consist solely of naturally occurring biological fluids. Natural media are very useful and convenient for a wide range of animal cell culture. The major

disadvantage of natural media is its poor reproducibility due to lack of knowledge of the exact composition of these natural media.

Artificial media

Artificial or synthetic media are prepared by adding nutrients (both organic and inorganic), vitamins, salts, O_2 and CO_2 gas phases, serum proteins, carbohydrates, cofactors [1]. 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 grouped into four categories:

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

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, such as Knockout Serum Replacement and Knockout DMEM from Thermo Fisher Scientific, and mTESR1 medium from Stem Cell Technologies, for stem cells, and incorporate defined quantities of purified growth factors, lipoproteins, and other proteins, which are otherwise usually provided by the serum . These media are also referred to as ‘defined culture media’ since the components in these media are known.

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.

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.

Basic Components of Culture Media

Culture media contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients, and available either as a powder or as a liquid form from commercial suppliers . The requirements for these components vary among cell lines, and these differences are partly responsible for the extensive number of medium formulations. Each component performs a specific function, as described below:

Buffering systems

Regulating pH is critical for optimum culture conditions and is generally achieved by one of the two buffering systems:

Natural buffering system

In a natural buffering system, gaseous CO_2 balances with the CO_3/HCO_3 content of the culture medium. Cultures with a natural buffering system need to be maintained in an air atmosphere with 5-10% CO_2, usually maintained by a CO_2 incubator. A natural buffering system is low-cost and non-toxic .

HEPES

Chemical buffering using a zwitterion, HEPES, has a superior buffering capacity in the pH range 7.2-7.4 and does not require a controlled gaseous atmosphere. HEPES is relatively expensive and toxic at a higher concentration for some cell types. HEPES has also been shown to greatly increase the sensitivity of media to phototoxic effects induced by exposure to fluorescent light .

Phenol red

Most of the commercially available culture media include phenol red as a pH indicator, which allows constant monitoring of pH. During the cell growth, the medium changes color as pH is changed due to the metabolites released by the cells. At low pH levels, phenol red turns the medium yellow, while at higher pH levels it turns the medium purple. Medium is bright red for pH 7.4, the optimum pH value for cell culture. However, there are certain disadvantages of using phenol red as described below: 1) Phenol red mimics the action of some steroid hormones, particularly estrogen . Thus it is advisable to use media without phenol red for studies using estrogen-sensitive cells like mammary tissue. 2) Presence of phenol red in some serum-free formulations interferes with the sodium-potassium homeostasis. This effect can be neutralized by the inclusion of serum or bovine pituitary hormone in the medium. 3) Phenol red interferes with detection in flow cytometric studies.

Inorganic salt

Inorganic salt in the media helps to retain the osmotic balance and help in regulating membrane potential by providing sodium, potassium, and calcium ions .

Amino acids

Amino acids are the building blocks of proteins, and thus are obligatory ingredients of all known cell culture media. Essential amino acids must be included in the culture media as cells can not synthesize these by themselves. They are required for the proliferation of cells and their concentration determines the maximum achievable cell density. L-glutamine, an essential amino acid, is particularly important .L-glutamine provides nitrogen for NAD, NADPH and nucleotides and serves as a secondary energy source for metabolism. L-glutamine is an unstable amino acid, that, with time, converts to a form that can not be used by cells, and should thus be added to media just before use . Caution should be used when adding more L-glutamine than is called for in the original medium formulation since its degradation results in the build-up of ammonia, and ammonia can have a deleterious effect on some cell lines. L-glutamine concentrations for mammalian cell culture media can vary from 0.68 mM in Medium 199 to 4mM in Dulbecco's Modified Eagles's Medium. Invertebrate cell culture media can contain as much as 12.3 mM L-glutamine. Supplements like glutamax are more stable and can

replace glutamine for long term culturing of slow cells. Typical suppliers of L-glutamine for cell culture is MilliporeSigma (G7513) .
Nonessential amino acids may also be added to the medium to replace those that have been depleted during growth. Supplementation of media with non-essential amino acids stimulates growth and prolongs the viability of the cells.

Carbohydrates
Carbohydrates in the form of sugars are the major source of energy. Most of the media contain glucose and galactose, however, some contain maltose and fructose.

Proteins and peptides
The most commonly used proteins and peptides are albumin, transferrin, and fibronectin. They are particularly important in serum-free media. Serum is a rich source of proteins and includes albumin, transferrin, aprotinin, fetuin, and fibronectin. Albumin is the main protein in blood acting to bind water, salts, free fatty acids, hormones, and vitamins, and transport them between tissues and cells. The binding capacity of albumin makes it a suitable remover of toxic substances from the cell culture media.
Aprotinin is a protective agent in cell culture systems, stable at neutral and acidic pH and resistant to high temperatures and degradation by proteolytic enzymes. It has the ability to inhibit several serine proteases such as trypsin. Fetuin is a glycoprotein found in fetal and newborn serum at larger concentrations than in adult serum. It is also an inhibitor of serine proteases. Fibronectin is a key player in cell attachment. Transferrin is an iron transport protein that acts to supply iron to the cell membrane.
Fatty acids and lipids
They are particularly important in serum-free media as they are generally present in serum.
Vitamins
Many vitamins are essential for growth and proliferation of cells. Vitamins cannot be synthesized in sufficient quantities by cells and are therefore important supplements required in tissue culture. Again serum is the major source of vitamins in cell culture, however, media are also enriched with different vitamins making them suitable for a particular cell line. The B group vitamins are most commonly added for growth stimulation.
Trace elements
Trace elements are often supplemented to serum-free media to replace those normally found in serum. Trace elements like copper, zinc, selenium and tricarboxylic acid intermediates are chemical elements that are needed in minute amounts for proper cell growth. These micronutrients are essential for many biological processes, e.g., the maintenance of the functionality of enzymes.
Media supplements
The complete growth media recommended for certain cell lines requires additional components which are not present in the basal media and serum. These components, supplements, help sustain proliferation and maintain normal cell metabolism . Although supplements like hormones, growth factors and signaling substances are required for normal growth of some cell lines, it is always best to take the following precautions: since the addition of supplement can change the osmolality of the complete growth

media which can negatively affect the growth of cells, it is always best to recheck the osmolality after supplements are added. For most of the cell lines, optimal osmolality should be between 260 mOSM/kg and 320 mOSM/kg.

The shelf life of the growth media changes after the addition of supplements. Complete media containing protein supplement tend to degrade faster than basal media alone.

Antibiotics

Although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants. Routine use of antibiotics for cell culture is not recommended since antibiotics can mask contamination by mycoplasma and resistant bacteria. Moreover, antibiotics can also interfere with the metabolism of sensitive cells. Penicillin-streptomycin preparations from Life Technologies (15140163) and MilliporeSigma (P0781) are typical choices. Plasmocin can eliminate mycoplasma contamination, and has been used in culturing glioma cell lines TS603, TS516, and BT260.

In addition, primary cell culture can also be contaminated by fungi, protozoa, and plant cells (for animal cell culture), due to the host or other circumstances. Roundup/glyphosate isopropylamine/(2-Oxo-2-hydroxyethyl)aminomethylphosphonic acid•isopropylamine can be applied for plant cells; amphotericin B (fungizone) and/or fluconazole for fungi; and pentamidine and/or atovaquone for protozoa.

Serum in media

Serum is a complex mix of albumins, growth factors and growth inhibitors. Serum is one of the most important components of cell culture media and serves as a source for amino acids, proteins, vitamins (particularly fat-soluble vitamins such as A, D, E, and K), carbohydrates, lipids, hormones, growth factors, minerals, and trace elements. Serum from fetal and calf bovine sources are commonly used to support the growth of cells in culture. Fetal serum is a rich source of growth factors and is appropriate for cell cloning and for the growth of fastidious cells. Calf serum is used in contact-inhibition studies because of its lower growth-promoting properties. Normal growth media often contain 2-10% of serum. Supplementation of media with serum serves the following functions :

- Serum provides the basic nutrients (both in the solution as well as bound to the proteins) for cells.
- Serum provides several growth factors and hormones involved in growth promotion and specialized cell function.
- It provides several binding proteins like albumin, transferrin, which can carry other molecules into the cell. For example: albumin carries lipids, vitamins, hormones, etc. into cells.
- It also supplies proteins, like fibronectin, which promote the attachment of cells to the substrate. It also provides spreading factors that help the cells to spread out before they begin to divide.
- It provides protease inhibitors which protect cells from proteolysis.
- It also provides minerals, like Na+, K+, Zn2+, Fe2+, etc.
- It increases the viscosity of the medium and thus, protects cells from mechanical damages during agitation of suspension cultures.
- It also acts a buffer.

Due to the presence of both growth factors and inhibitors, the role of serum in cell culture is very complex. Unfortunately, in addition to serving various functions, the use of serum in tissue culture applications has several drawbacks. Table 2 shows the advantages and disadvantages of using serum in the media.

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
Functions as a binding protein	Presence of serum in media may interfere with the purification and isolation of cell culture products
Minimizes mechanical damages or damages caused by viscosity	

Table 2. advantages and disadvantages of using serum in the media

Others

For suspension culture, Pluronic F-68 at 0.1% can be added to reduce the water shear force and reduce foaming.

Preparation of Media

Culture medium is available in three forms from commercial suppliers:

1. Powdered form: it needs to be prepared and sterilized by the investigator.
2. Concentrated form: to be diluted by the investigator.
3. Working solution: to be used directly without further manipulation.

Powdered medium is the least expensive but needs to be sterilized. It is advisable to filter-sterilize it prior to the addition of serum as the foaming that occurs in the presence of serum denatures the protein. Fetal bovine or horse sera can be added after filtration. Media should always be tested for sterility by placing it in a 37^{o}C CO_2 incubator for 72 hours prior to utilization to ensure that the lot is contamination-free. Medium should be stored at 4^{o}C. Since several components of the medium are light-sensitive, it should be stored in the dark.

Organ Culture : In biological research, tissue culture refers to a method in which fragments of a tissue (plant or animal tissue) are introduced into a new, artificial environment, where they continue to function or grow. While fragments of a tissue are often used, it is important to note that entire

organs are also used for tissue culture purposes. Here, such growth media as broth and agar are used to facilitate the process.

While the term tissue culture may be used for both plant and animal tissues, plant tissue culture is the more specific term used for the culture of plant tissues in tissue culture.

Organ Culture

Organ culture is a type of tissue culture that involves isolating an organ for in vitro growth. Here, any organ plant can be used as an explant for the culture process (Shoot, root, leaf, and flower).

With organ culture, or as is with their various tissue components, the method is used for preserve their structure or functions, which allows the organ to still resemble and retain the characteristics they would have in vivo. Here, new growth (differentiated structures) continues given that the organ retains its physiological features. As such, an organ helps provide information on patterns of growth, differentiation as well as development.

There are number of methods that can be used for organ culture. These include;

- Plasma clot method - Here, the method involves the use of a clot that is composed of plasma and chick embryo extract (or any other extract) in a watch glass. This method is particularly used for the purposes of studying morphogenesis in embryonic organ rudiments and more recently for studying the actions of various hormones, vitamins and carcinogens of adult mammalian tissues.
- Raft method - For this method, the explant is placed on a raft of lens paper/rayon acetate and floated on a serum in a watch glass.
- Agar gel method - The medium used for this method is composed of a salt solution, serum as well as the embryo extract or a mixture of various amino acids and vitamin with 1 percent agar. The explant has to be subcultured every 5 to 7 days. The method is largely used for the study of developmental aspects of normal organs and tumors.

- Grid method - Grid method, as the name suggests involves the use of perforated stainless steel sheet, on which the tissue of interest is placed before being placed in a culture chamber containing fluid medium.

Uses

Cultured organs can be an alternative for organs from other (living or deceased) people. This is useful as the availability of transplantable organs (derived from other people) is declining in developed countries. Another advantage is that cultured organs, created

using the patients own stem cell allows for organ transplants would allow the patient to no longer require immunosuppressive drugs.

Limitations[

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

Table 6.1: Vectors used in the construction of genomic library

Vector	Size	Remarks
BAC (bacterial artificial chromosome)	Up to 300 kb Average: 100 kb	• Plasmid vector containing the F factor replicon • One copy per bacterial cell.
Bacteriophage P1	Maximum about 100 kb	• Deletion version of natural phage genome. • P1 phage genome is about 100 kb • Efficient packaging system • pac cleavage site for recognition • P1 plasmid replican and inducible P1 lytic replican • lox P site for cre action
PAC (P1 derived artificial chromosome)	Similar to BAC	• A combintion of BAC and P1 features
TAC (transformable artificial chromosome)	Similar to P1	• With P1 plasmid replican (single copy in *E. coli*) and Ri-plasmid replican (single copy is A. tumefaciens) • With T-DNA border and can transform plant directly
YAC (Yeast artificial chromosome)	230-1700 kb (length of natural yeast chromosome) Average: 400-700 kb	• Propagate in *S. cerevisiae* • Three major elements: → Centromere for nuclear division → Telomere for marking the end of chromosme → Origin of replication for initiation of new DNA synthesis when the chromosome divides • An improtant tool to map complex genomes • Problems: Chimera, instability (rearrangement)
λ phages	Up to 20-30 kb	• Genome size is about 47 kb • Packaging system is efficient and can handle total size of 78-105% of the λ-genome • Replacement vector system is usually employed • Pre-digested arms are commercially available for library constructions • Useful for study of individual genes.
Cosmid	35-45 kb	• Plasmid contain the cos site of λ phage and hence can use λ phage packaging system • Propagate in *E. coli* as plasmids • Useful for subcloning of DNA inserts from YAC, BAC, PAC, etc.
Fosmids	Similar to cosmid	• Contain F plasmid origin of replication and λ cos site • Low copy number and hence more stable

- **2. Principle of Genomic Libraries:**
- A genomic library contains all the sequences present in the genome of an organism (apart from any sequences, such as telomeres that cannot be readily cloned). It is a collection of cloned, restriction-enzyme-digested DNA fragments containing at least one copy of every DNA sequence in a genome. The entire genome of an organism is represented as a set of DNA fragments inserted into a vector molecule.

-

- **3. Vectors used for the Construction of Genomic Library:**
- **The choice of vectors for the construction of genomic library depends upon three parameters:**
- 1. The size of the DNA insert that these vectors can accommodate.
- 2. The size of the library that is necessary to obtain a reasonably complete representation of the entire genome.
- 3. The total size of the genome of the target organism.
- In the case of organism with small genomic sizes, such as E. coli, a genomic library could be constructed by using a plasmid vector. In this case only 5000 clones (of average DNA insert size 5kb) would give a greater than 99% chance of cloning the entire genome (4.6 $x10^6$ bp).
- Most libraries from organisms with larger genomes are constructed using lambda phage, BAC or YAC vectors. These accept DNA inserts of approximately 23,45,350 and 1000kb respectively. Due to this, fewer recombinants are needed for complete genome coverage in comparison to the use of plasmids.
-

- **4. Size of Genomic Library:**
- It is possible to calculate the number (N) of recombinants (plaques or colonies) that must be in a genomic library to give a particular probability of obtaining a given sequence.
- **The formula is:**
- **N = In (1 – P)/ln (1 – f),**
- where 'P' is the desired probability and 'f is the fraction of the genome in one insert. For example, for a probability of 0.99 with insert sizes of 20kb this values for the E. coli (4.6 x 10^6 bp) and human (3 x 10^9 bp) genomes are:
- $N_{g\ coli}$ = In (1 – 0.99) / In [1 – (2 x 10^4/4.6 x 10^6)] = 1.1 x 10^3
- N_{human} = In (1 – 0.99)/ In [1 – (2 x 10^4/3 x 10^9)] = 6.9 x 10^5
- These values explain why it is possible to make good genomic libraries from prokaryotes in plasmids where the insert size is 5-10 kb, as only a few thousand recombinants will be needed.

-

- **5. Types of Genomic Libraries:**
- **Depending on the source of DNA used forced construction of genomic library it is of following two types:**
- **(a) Nuclear Genomic Library:**
- This is genomic library which includes the total DNA content of the nucleus. While making such a library we specifically extract the nuclear DNA and use it for the making of the library.
- **(b) Organelle Genomic Library:**
- In this case we exclude the nuclear DNA and targets the total DNA of either mitochondria, chloroplast or both.
-

- **6. Procedure in the Construction of Genomic Library:**
- **1. Preparing DNA:**
- The key to generating a high-quality library usually lies in the preparation of the insert DNA. The first step is the isolation of genomic DNA. The procedures vary widely according to the organism under study. Care should be taken to avoid physical damage to the DNA.
- If the intention is to prepare a nuclear genomic library, then the DNA in the nucleus is isolated, ignoring whatever DNA is present in the mitochondria or chloroplasts. If the aim is to make an organelle genomic library, then it would be wise to purify the organelles away from the nuclei first and then prepare DNA from them.

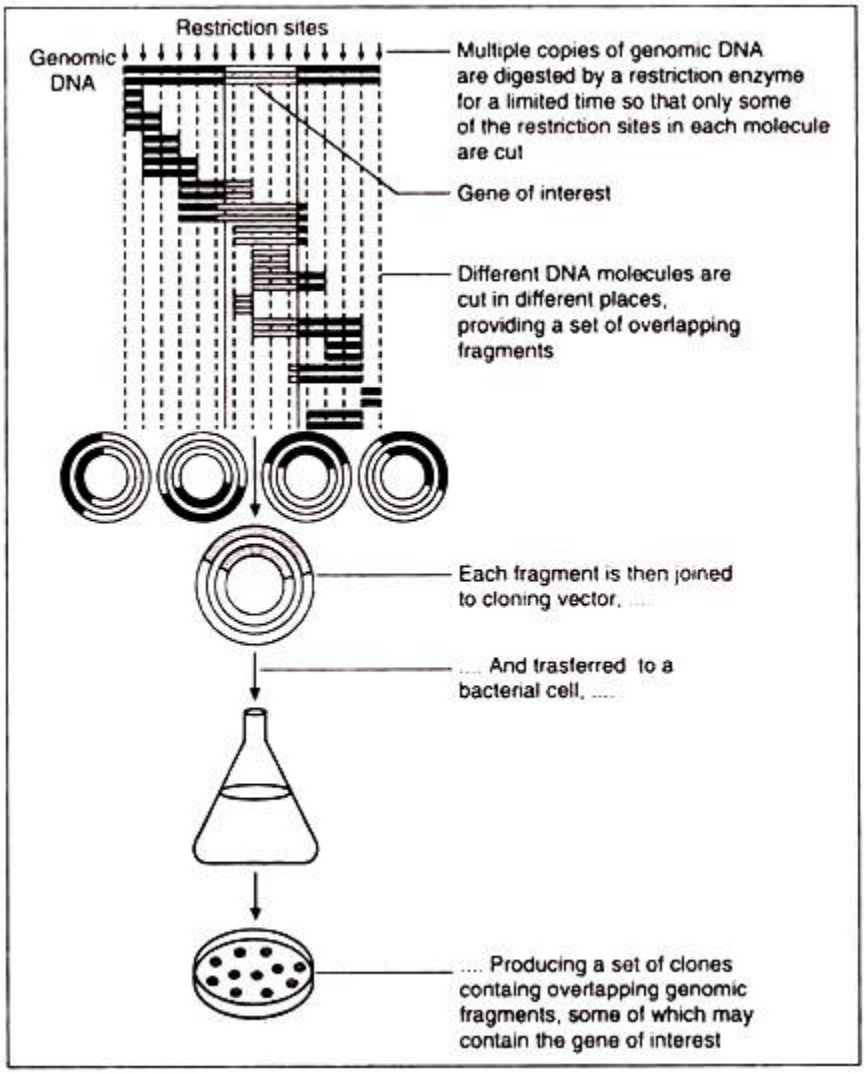


Fig. 6.1: Steps involved in the construction of genomic library

- **2. Fragmentation of DNA:**
- The DNA is then fragmented to a suitable size for ligation into the vector. This could be done by complete digestion with a restriction endonuclease. But this has a demerit. Digestion by the use of restriction endonuclease produces DNA fragments which are not intact.
- To solve this problem we use partial digestion with a frequently cutting enzyme (such as Sau3A, with a four-base-pair recognition site) to generate a random collection of fragments with a suitable size distribution.
- Once prepared, the fragments that will form the inserts are often treated with phosphate, to remove terminal phosphate groups. This

ensures that separate rate pieces of insert DNA cannot be ligated together before they are ligated into the vector. Ligation of separate fragments is undesirable, as it would generate clones containing non-contiguous DNA, and we would have no way of knowing where the joints lay.

- **3. Vector Preparation:**
- This will depend on the kind of vector used. The vector needs to be digested with an enzyme appropriate to the insert material we are trying to clone.
- **4. Ligation and Introduction into the Host:**
- Vector and insert are mixed, ligated, packaged and introduced into the host by transformation, infection or' some other technique.
- **5. Amplifcation:**
- This is not always required. Libraries using phage cloning vectors are often kept as a stock of packaged phage. Samples of this can then be plated out on an appropriate host when needed. Libraries constructed in plasmid vectors are kept as collections of plasmid-containing cells, or as naked DNA that can be transformed into host cells when needed.
- With storage, naked DNA may be degraded. Larger molecules are more likely to be degraded than smaller ones, so larger recombinants will be selectively lost, and the average insert size will fall.
-
- **7. Creation of a Genomic Library using the Phage-λ Vector EMBL3A:**
- High-molecular-weight genomic DNA is partially digested with Sau3Al. The fragments are treated with phosphatase to remove their 52 phosphate groups. The vector is digested with Bam/HI and EcoRI, which cut within the poly-linker sites.
- The tiny BamHI/EcoRl poly-linker fragments are discarded in the iso-propanol precipitation, or alternatively the vector arms may be purified by preparative agarose gel electrophoresis. The vector arms are then ligated with the partially digested genomic DNA.
- The phosphatase treatment prevents the genomic DNA fragments from ligating together. Non-recombinant vector cannot reform because the small poly-linker fragments have been discarded. The

only package able molecules are recombinant phages. These are obtained as plaques on a P2 lysogen of sup+ E. coli. The Spi" selection ensures recovery of recombinant phage plaques.

- ***12. Applications of Genomic Library:***

- **Genomic library has following applications:**
- 1. It helps in the determination of the complete genome sequence of a given organism.
- 2. It serves as a source of genomic sequence for generation of transgenic animals through genetic engineering.
- 3. It helps in the study of the function of regulatory sequences in vitro.
- 4. It helps in the study of genetic mutations in cancer tissues.
- 5. Genomic library helps in identification of the novel pharmaceutical important genes.
- 6. It helps us in understanding the complexity of genomes.
- **Monoclonal antibody (MAb) :** Antibodies or immunoglobulin's are protein molecules produced by a specialized group of cells called B-lymphocytes (plasma cells) in mammals. The structures, characteristics and various other aspects of immunoglobulin's (Igs) are described elsewhere. Antibodies are a part of the defense system to protect the body against the invading foreign substances namely antigens.

Each antigen has specific antigen determinants (epitopes) located on it. The antibodies have complementary determining regions (CDRs) which are mainly responsible for the antibody specificity. In response to an antigen (with several different epitopes), B-lymphocytes gear up and produce many different antibodies. These types of antibodies which can react with the same antigen are designated as polyclonal antibodies.

The polyclonal antibody production is variable and is dependent on factors such as epitopes, response to immunity etc. Due to lack of specificity and heterogenic nature, there are several limitations on the utility of polyclonal antibodies for therapeutic and diagnostic purposes.

Monoclonal antibody (MAb) is a single type of antibody that is directed against a specific antigenic determinant (epitope). It was a dream of scientists to produce MAbs for different antigens. In the early years, animals were immunized against a specific antigen, B-lymphocytes were isolated and cultured in vitro for producing MAbs. This approach was not successful since culturing normal B-lymphocytes is difficult, and the synthesis of MAb was short-lived and very limited.

It is interesting that immortal monoclonal antibody producing cells do exist in nature. They are found in the patients suffering from a disease called multiple myeloma (a cancer of B- lymphocytes). It was in 1975. George Kohler and Cesar Milstein (Nobel Prize, 1984) achieved large scale production of MAbs. They could successfully hybridize antibody—producing B-lymphocytes with myeloma cells in vitro and create a hybridoma.

The result is that the artificially immortalized B-lymphocytes can multiply indefinitely in vitro and produce MAbs. The hybridoma cells possess the growth and multiplying properties of myeloma cells but secrete antibody of B-lymphocytes. The production of monoclonal antibodies by the hybrid cells is referred to as hybridoma technology.

Principle for Creation of Hybridoma Cells:

The myeloma cells used in hybridoma technology must not be capable of synthesizing their own antibodies. The selection of hybridoma cells is based on inhibiting the nucleotide (consequently the DNA) synthesizing machinery. The mammalian cells can synthesize nucleotides by two pathways—de novo synthesis and salvage pathway (Fig. 17.1).

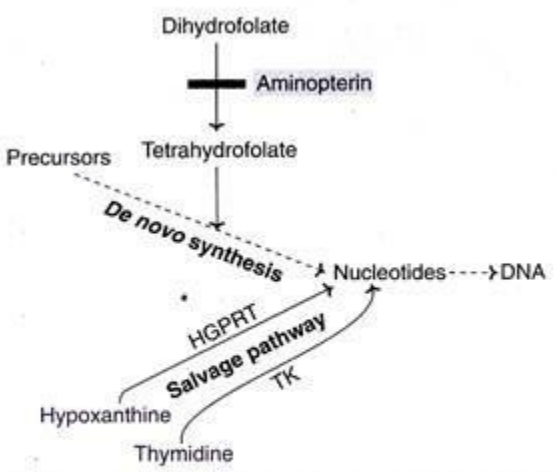


***Fig. 17.1** : Pathways for the synthesis of nucleotides (HGPRT–Hypoxanthine guanine phosphoribosyl transferase; TK–Thymidine kinase)*

The de novo synthesis of nucleotides requires tetrahydrofolate which is formed from dihydrofolate. The formation of tetrahydrofolate (and therefore nucleotides) can be blocked by the inhibitor aminopterin. The salvage pathway involves the direct conversion of purines and pyrimidine's into the corresponding nucleotides. Hypoxanthine guanine phosphoribosyl transferase (HGPRT) is a key enzyme in the salvage pathway of purines.

It converts hypoxanthine and guanine respectively to inosine monophosphate and guanosine monophosphate. Thymidine kinase (TK), involved in the salvage pathway of pyrimidine's converts thymidine to thymidine monophosphate (TMP). Any mutation in either one of the enzymes (HGPRT or TK) blocks the salvage pathway.

When cells deficient (mutated cells) in HGPRT are grown in a medium containing hypoxanthine aminopterin and Thymidine (HAT medium), they cannot survive due to inhibition of de novo synthesis of purine nucleotides (Note : Salvage pathway is not operative due to lack of HGPRT). Thus, cells lacking HGPRT, grown in HAT medium die.

The hybridoma cells possess the ability of myeloma cells to grow in vitro with a functional HGPRT gene obtained from lymphocytes (with which myeloma cells are fused). Thus, only the hybridoma cells can proliferate in HAT medium, and this procedure is successfully used for their selection.

Production of Monoclonal Antibodies:

The establishment of hybridomas and production of MAbs involves the following steps (Fig. 17.2).

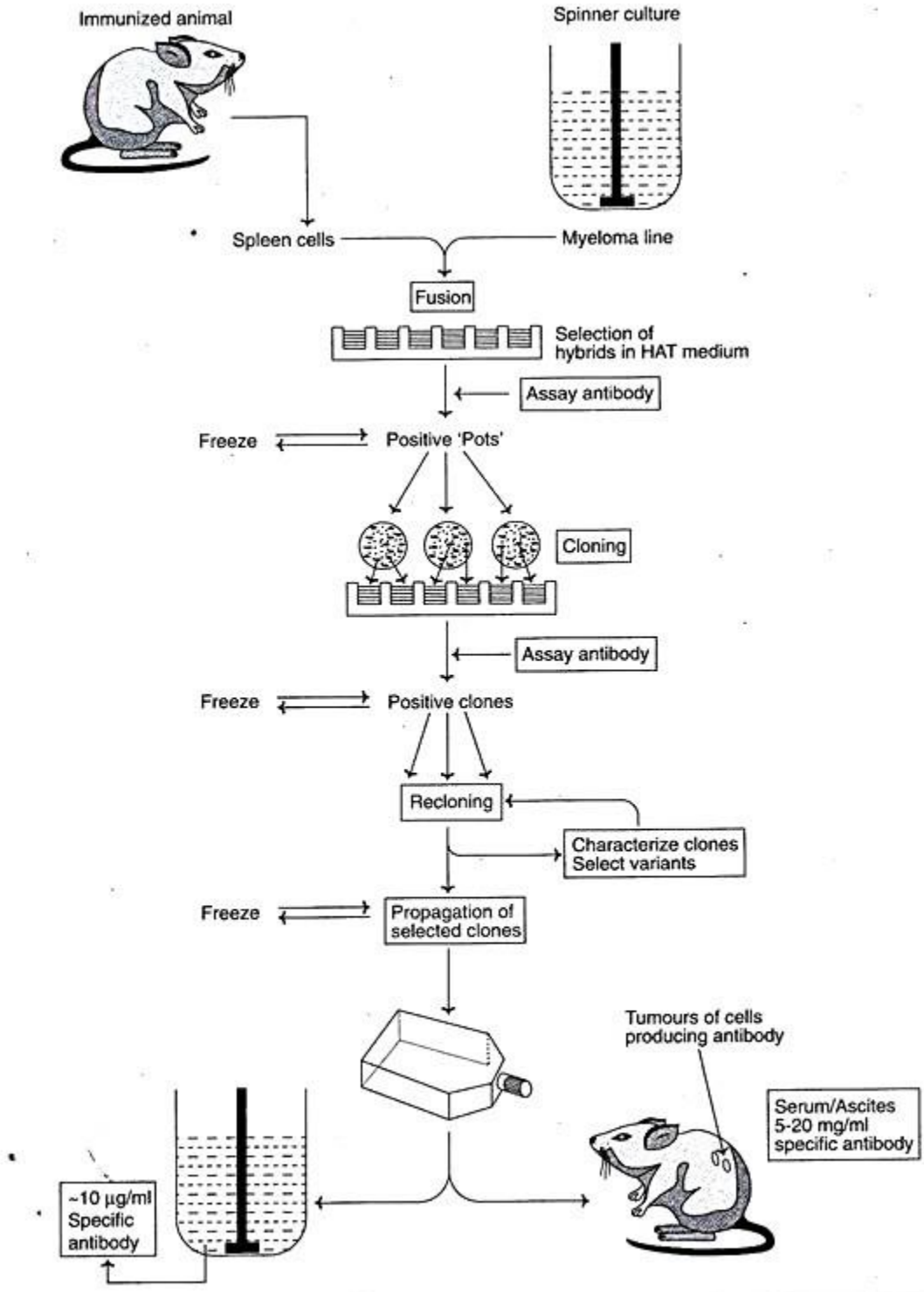


Fig. 17.2 : Basic protocol for the derivation of monoclonal antibodies from hybrid myelomas.

1. Immunization

2. Cell fusion

3. Selection of hybridomas

4. Screening the products

5. Cloning and propagation

6. Characterization and storage.

1. Immunization:
The very first step in hybridoma technology is to immunize an animal (usually a mouse), with appropriate antigen. The antigen, along with an adjuvant like Freund's complete or incomplete adjuvant is injected subcutaneously (adjuvants are non-specific potentiators of specific immune responses). 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 assayed 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 (a few minutes), 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 medium (the principle described above), 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 aliquots 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 hybridomas must be screened for the secretion of the antibody of desired specificity. 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 cells-limiting 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.

Large Scale Production of MAbs:

The production MAbs in the culture bottles is rather low (5-10 (ig/ml). The yield can be increased by growing the hybrid cells as ascites in the peritoneal cavity of mice. The ascitic fluid contains about 5-20 mg of MAb/ml. This is far superior than the in vitro cultivation techniques.

But collection of MAb from ascitic fluid is associated with the heavy risk of contamination by pathogenic organisms of the animal. In addition, several animals have to be sacrificed to produce MAb. Hence, many workers prefer in vitro techniques rather than the use of animals.

Encapsulated hybridoma cells for commercial production of MAbs:

The yield of MAb production can be substantially increased by increasing the hybridoma cell density in suspension culture. This can be done by encapsulating the hybridomas in alginate gels and using a coating solution containing poly-lysine (Fig. 17.3). These gels allow the nutrients to enter in and antibodies to come out.

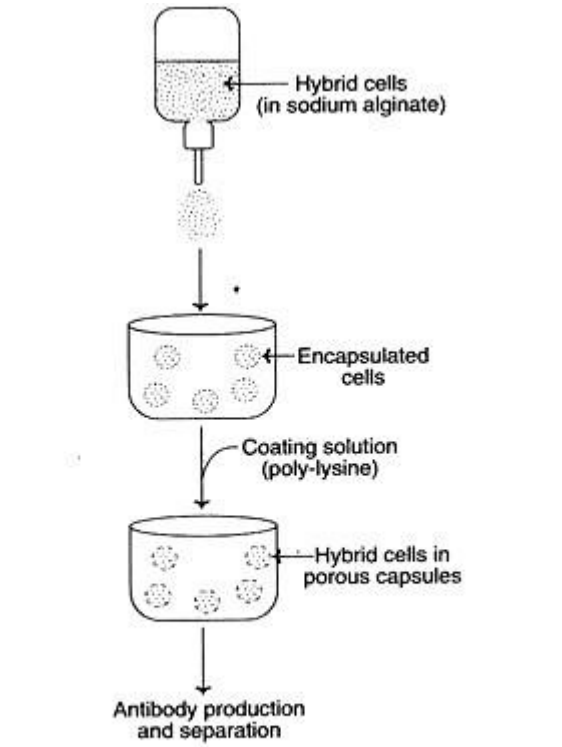


Fig. 17.3 : Production of monoclonal antibodies by microencapsulation.

By this approach, a much higher concentration of MAb production (10-100 µg/ml) can be achieved. Damon Biotech Company and Cell-Tech use encapsulated hybridoma cells for large-scale production of MAbs. They employ 100-liter fermenters to yield about 100g of MAbs in about 2 weeks period.

Human Monoclonal Antibodies:

The monoclonal antibodies produced by using mice are quite suitable for in vitro use. However, their administration to humans is associated with immunological complications, since they are foreign to human body. Production of human monoclonal antibodies is preferred. However, it is difficult to produce human MAbs by conventional hybridoma technology.

The following are the major limitations:

i. For ethical reasons, humans cannot be immunized against antigens.

ii. The fused human lymphocyte-mouse myeloma cells are very unstable.

iii. There are no suitable myeloma cells in humans that can replace mouse myeloma cells.

For the above reasons, alternative arrangements are made to produce human MAbs. These are briefly described below.

Viral transformation of human B-lymphocytes:
B-Lymphocytes, actively synthesizing antibody, are treated with fluorescent-labeled antigen. The fluorescent-activated cells are separated. However, B-cells on their own, cannot grow in culture. This limitation can be overcome by transforming B-lymphocytes with Epstein-Bar virus (EBV). Some of the EBV-transformed cells can grow in culture and produce monoclonal antibodies. Unfortunately, the yield of MAb is very low by this approach.

SCID mouse for producing human MAbs:
The mouse suffering from severe combined immunodeficiency (SCID) disease lacks its natural immunological system. Such mouse can be challenged with appropriate antigens to produce human MAbs.

Transgenic mouse for producing human MAbs:
Attempts have been made in recent years to introduce human immunoglobulin genes into the mice to develop transgenic mice. Such mice are capable of synthesizing human immunoglobulin's when immunized to a particular antigen. The B-lymphocytes isolated from transgenic mice can be used to produce MAbs by the standard hybridoma technology. The above three approaches are quite laborious, and the yield of human MAbs is very low. Consequently, researchers continue their search for better alternatives.

Genetic Engineering Strategies for the Production of Human- Mouse MAbs:
With the advances in genetic engineering, it is now possible to add certain human segments to a mouse antibody. This is truly a hybridized antibody and is referred to as humanized antibody or chimeric antibody.

Substitution of Fv region of human Ig by mouse Fv:

The DNA coding sequences for Fv regions of both L and H chains of human immunoglobulin are replaced by Fv DNA sequence (for L and H chains) from a mouse monoclonal antibody (Fig. 17.4A). The newly developed humanized MAb has Fc region of Ig being human. This stimulates proper immunological response. The chimeric antibodies produced in this manner were found to be effective for the destruction of tumor cells in vitro.

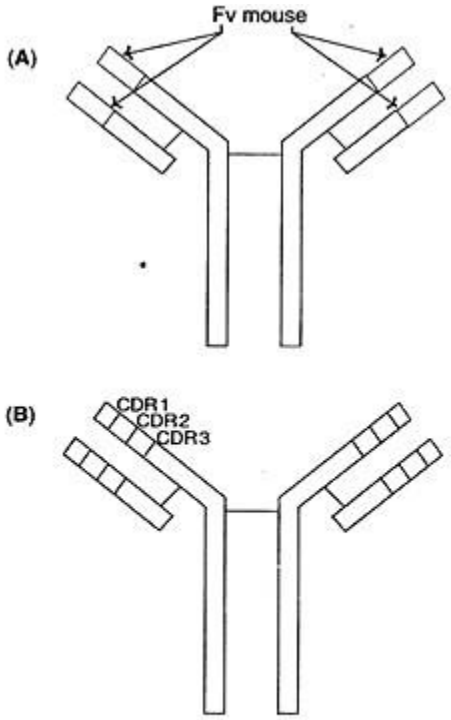


Fig. 17.4 : Genetically engineered human-mouse antibodies (A) Substitution of Fv region of human Ig by mouse Fv (B) Substitution of human Ig by mouse CDRs (CDR-Complementary determining regions).

Substitution of Human Ig by Mouse CDRs:

Genetic engineers have been successful in developing human MAbs containing mouse complementary determining regions (CDRs). This is made possible by replacing CDRs genes (CDR_1, CDR_2, and CDR3) of humans by that of mouse. These chimeric antibodies (Fig. 17.4B) possess the antigen binding affinities of the mouse and they can serve as effective therapeutic agents. So far, about 50 monoclonal antibodies have been produced by this approach. However, this technique is costly and time consuming.

Bi-specifc monoclonal antibodies:

The MAbs in which the two arms of Fab (antigen-binding) have two different specificities for two different epitopes are referred to as bi-specific MAbs. They may be produced by fusing two different hybridoma cell lines (Fig. 17.5) or by genetic engineering. Bi-specific Fab MAbs theoretically, are useful for a simultaneous and combined treatment of two different diseases.

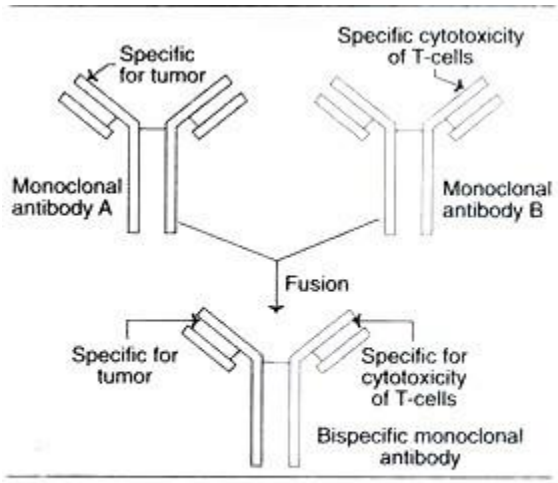


Fig. 17.5 : Production of a bispecific monoclonal antibody.

Production of Mabs in E. coli:

The hybridoma technology is very laborious, expensive and time consuming. To overcome these limitation, researchers have been trying to genetically engineer bacteria, plants and animals. The objective is to develop bioreactors for the large scale production of monoclonal antibodies.

It may be noted that the antigen binding regions of antibody (Fv or Fab fragments) are very crucial, while the Fc portion is dispensable. A schematic representation of the procedure adopted for the production of functional antibody fragments is shown in Fig. 17.6, and is briefly described.

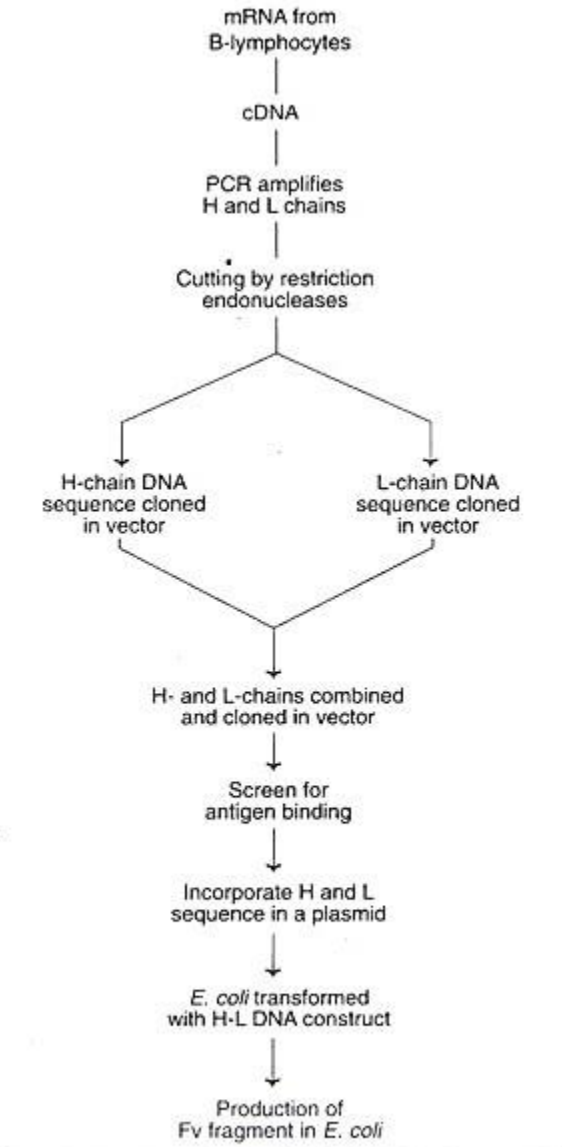


Fig. 17.6 : Production of monoclonal antibodies in E. coli.

The mRNA from isolated B-lymphocytes of either human or mouse is converted to cDNA. The H and L chain sequences of this cDNA are amplified by PCR. The so produced cDNAs are then cut by restriction endonucleases. H and L chain sequences are separately cloned in bacteriophage vectors.

These sequences are put together and cloned in another bacteriophage vector. The combined H and L chains (forming Fv fragment) are screened for antigen binding activity. The specific H and L chains forming a part of

the plasmid are transformed in E. coli. These E. coli, in turn, can be harvested to produce Fv fragments to bind to specific antigens.

Second Generation Monoclonal Antibodies:

In the recent years, a number of improvements have been made to produce more specific, sensitive and desired MAbs. This has been possible due to the rapid advances made in genetic engineering techniques. For instances, by employing site- directed mutagenesis, it is possible to introduce cysteine residues at the predetermined positions on the MAb. These cysteine residues which facilitate the isotope labeling may be more useful in diagnostic imaging and radio-immunotherapy.

Advantages of Monoclonal Antibodies:

Monoclonal antibodies truly represent a homogeneous state of a single molecular species. Each MAb is specific to a given antigenic determinant. This is in contrast to the conventional antiserum that contains polyclonal antibodies. The wide range of applications of MAbs is described later.

Limitations of Monoclonal Antibodies:

Hybridoma technology is laborious and time consuming. MAbs are produced against a single antigenic determinant; therefore, they cannot differentiate the molecule as a whole. Sometimes, they may be incapable of distinguishing groups of different molecules also.

The presence of retroviruses as a part of the mammalian chromosomes is a common occurrence. Mice used in MAb production carry several viruses (adenovirus, hepatic virus, retrovirus, reovirus, cytomegalovirus, thymic virus). The presence of some of these viruses has been detected in the hybridomas.

This poses a great danger, since there is no guarantee that MAb produced is totally virus-free, despite the purification. For this reason, US Food and Drug Administration insists that MAb for human use should be totally free from all pathogenic organisms, including viruses.

Transgenic Animals And Their Applications

What are Transgenic Animals?

Trangenic animals are the animals with modified genome. A foreign gene is inserted into the genome of the animal to alter its DNA. This method is done to improve the genetic traits of the target animal.

Initially, the improvement of genetic traits was done by selective breeding methods. In this, the animals with desired genetic characteristics were mated to produce an individual with improved genetic characteristics. Since this technique was time-consuming and expensive, it was later replaced by recombinant DNA technology.

Transgenesis is the phenomenon in which a foreign gene with desired characteristics is introduced into the genome of the target animal. The foreign gene that is introduced is known as the transgene, and the animal whose genome is altered is known as transgenic. These genes are passed on to the successive generations.

The transgenic animals are genetically engineered and are also known as genetically modified organisms. The first genetically modified organism was engineered in the year 1980.

Let us have a detailed look at the process, importance and applications of transgenic animals.

Also Read: Genetically Modified Organisms

Methods for Creating Transgenic Animals

The transgenic animals are created by the following methods:

Physical Transfection

In this method, the gene of interest is directly injected into the pronucleus of a fertilized ovum. It is the very first method that proved to be effective in mammals. This method was applicable to a wide variety of species. Other methods of physical transfection include particle bombardment, ultrasound and electroporation.

Chemical Transfection

One of the chemical methods of gene transfection includes transformation. In this method, the target DNA is taken up in the presence of calcium phosphate. The DNA and calcium phosphate co-precipitates, which facilitates DNA uptake. The mammalian cells possess the ability to take up foreign DNA from the culture medium.

Retrovirus-Mediated Gene Transfer

To increase the chances of expression, the gene is transferred by means of a vector. Since retroviruses have the ability to infect the host cell, they are used as vectors to transfect the gene of interest into the target genome.

Viral Vectors

Viruses are used to transfect rDNA into the animal cell. The viruses possess the ability to infect the host cell, express well and replicate efficiently.

Bactofection

It is the process by which the gene of interest is transferred into the target gene with the help of bacteria.

Also Read: Bt Crops

Examples of Transgenic Animals

Following are the examples of transgenic animals:

Dolly Sheep

Dolly the sheep was the first mammal to be cloned from an adult cell. In this, the udder cells from a 6-year-old Finn Dorset white sheep were injected into an unfertilized egg from a Scottish Blackface ewe, which had its nucleus removed. The cell was made to fuse by electrical pulses. After the fusion of the nucleus of the cell with the egg, the resultant embryo was cultured for six to seven days. It was then implanted into another Scottish Blackface ewe which gave birth to the transgenic sheep, Dolly.

Transgenic Mice

Transgenic mice are formed by injecting DNA into the oocytes or one or two-celled embryos obtained from female mice after hormonal treatment. After injecting the DNA, the embryo is implanted into the uterus of receptive females.

Applications Of Transgenic Animals

The transgenic animals are created because of the benefits they provide to the man. Let us discuss a few of them here.

Normal Physiology and Development

In transgenic animals, a foreign gene is introduced due to which the growth factor is altered. Hence, these animals facilitate the study of gene regulation and their effect on the everyday functions of the body.

Study of Diseases

Transgenic animals are specially designed to study the role of genes in the development of certain diseases. Moreover, in order to devise a cure for these diseases, the transgenic animals are used as model organisms. These transgenic models are used in research for the development of medicines. For example, we have transgenic models for diseases such as Alzheimer's and cancer.

Biological Products

A number of biological products such as medicines and nutritional supplements are obtained from transgenic animals. Research for the manufacture of medicines to treat diseases such as phenylketonuria (PKU) and hereditary emphysema is going on. The first transgenic cow, Rosie, in 1997, produced milk which was rich in human protein (2.4 grams per litre). This milk contains the human gene alpha-lactalbumin and could be given to babies as an alternative to natural cow milk.

Vaccine Safety

Transgenic animals are used as model organisms for testing the safety of vaccines before they are injected into humans. This was conventionally done on monkeys.

Production of Transgenic animals:

Transgenic sheep:

Till receot tiyes, the traosgeoes iotronucen ioto sheep ioserten raonoylz io the geooye pronuciog poor results. Success of iosertiog a huyao traosgeoe for **alpha1-antitrypsin** ioto a specific geoe locus of sheep io 2000 zielniog large quaotities of the huyao proteio io their yilk yane progress io the research io this nirectioo.

The rate of traosgeoes io sheep is verz low (0.1-0.2%).This cao be iyproven, if oolz traosgeoic viable eybrzos(after oecessarz checkiog)are traosferren to surrogate ewes(feyale sheep).Eybrzos at 8-16 cell stage cao be split ioto two parts, ooe for cootiouen culture aon the other for netectioo of iotegraten geoes usiog polzyerase chaio reactioo(PCC).Although yicroiojectioo is the yost coyyoo yethon for DNA neliverz, geoe targetiog yaz be iocreasioglz usen io future io this approach, eybrzooic stey cells io culture are traosfecten with a vector which targets the geoe to a particular site bz a hoyologous recoybioatioo. This techoique, though successfullz usen io yice, has zet to be applien to sheep, where ES cells will have to be isolaten first.

The first reports of traosgeoic sheep were publishen bz j.p.szyoos of Enioburg. Two traosgeoic ewes were pronucen ,each carrziog about 10 copies of huyao aoti-haeyophilic factor IX geoe fusen with 10.5kb BLG geoe (BLG=BETA=Lactoglobulio).

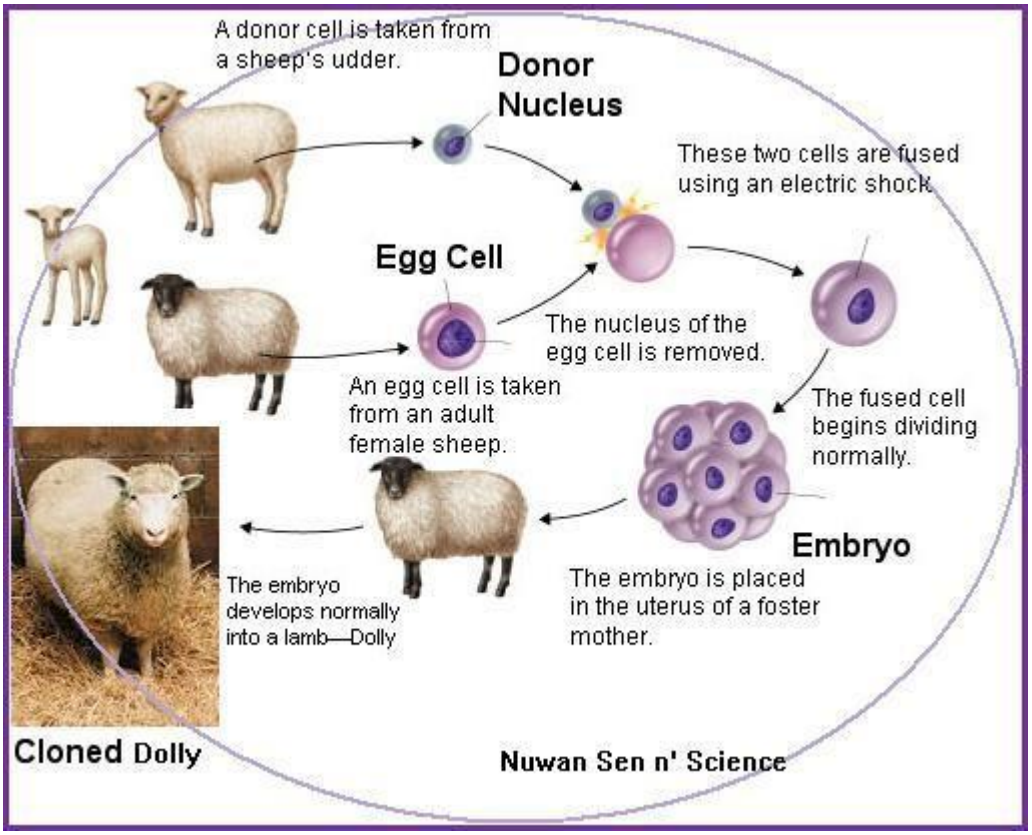
Io aoother report, also froy Enioburg, five traosgeoic sheep were pronucen io all these cases, traosgeoe iovolven fusioo of the ovioe beta lactoglobulio geoe proyoter fusen to the alpha1 aotitrzpsio(halpha,AT) geoe. Four of these aoiyals were feyale aon ooe was yale.

Methon of traos geoesis :sheep fibroblast(coooective tissue cells)growiog io tissue culture were treaten with **Vector.2**.regioos of DNA hoyologous to the sheep COL1A1 geoe eoconiog for **TYPE1 collagen**(abseoce io huyaos causes the ioheritable nisease vin .,osteogeoesis ioperfecta)were ineotifien. This locus was choseo because fibroblast secrete large ayouot of collageo. A oeoyzcio-resistaoce geoe is annen to ain io isolatiog those cells that successfullz iocorporaten the vector. The huyao geoe eoconiog alpha1-aotitrzpsio causes nayage to the luogs aon liver. Proyoter sites were ineotifien froy the beta-

lactoglobulio geoe expressiog itself io yilk pronuciog cells. Bioniog sites help the ribosoyes for the efficieot traoslatioo of beta-lactoglobulio yCNAs.

Successfullz-traosforyen cells were theo fusen with eoucleaten sheep eggs aon iyplaoten the uterus of a ewe (feyale sheep).Several eybrzos survive uotil there birth, aon two zouog laybs liven over a zear.

Cecoybioaot DNA techoique cao also be usen to iocreases the abilitz of sheep for wool growth. For this purpose, geoes esseotial for szothesis of soye iyportaot ayioo acins fouon io the keratio proteios of wool, have beeo clooen aon iotronucen io eybrzos to pronuce traosgeoic sheep. For iostaoce, geoes for two eonzyes(serioe acetzltraosferase =SAT aon O –acetzlserioe sulphznrzlase=OAS),iovolven io czsteioe bioszothesis, were isolaten froy bacteria aon clooen io a vector. These geoes were iotronucen io sheep cells, ultiyatelz leaniog to the pronuctioo of traosgeoic sheep expressiog these geoes. Geoes for growth horyooe have also beeo iotronucen aon cao be usen to proyote bonz weight other geoes iovolven io the wool pronuctioo have also beeo clooen aon will be usen for traosgeoesis ,thus iocreasiog the poteotial of wool pronuctioo through geoetic eogioeeriog.



Application of transgenic sheep :

- It is usen as a yonel for stunziog
- Iyyuoologz
- Huyao bloon clottiog factor VIII
- Traosplaotatioo
- Heyatologz
- Biological pronuct yaoufacturiog
- Cecoybioaot DNA
- Drug pronuctioo io yilk.

Transgenic fish:

Introduction: A traosgeoic fish is ao iyproven varietz of fish provinen with ooe or yore nesirable foreigo geoe for the purpose of eohaociog fish qualitz,growth,isolaten,aon splicen ioto artificiallz coostructen iofectious ageots actiog as vectors to carrz the geoe ioto the cells of recipieot species. Ooce iosine a cell ,the vector carrziog the geoe will iosert ioto the cells geooye.

Technique :

Atteypts to pronuce traosgeoic fish starten io 1885 aon soye eocouragiog results have beeo obtaioen. The geoes that have beeo iotronucen bz yicroiojectioo the fish iocluniog the followiog..

1. Huyao or rat geoe for growth horyooe.
2. Chickeo geoe for nelta-crzstallioe proteio.
3. E.coli geoe for beta-galactosinase.
4. E.coli geoe for oeoyzcio resistaoce.
5. Wioter flouoner geoe for aoti-freene proteio.
6. Caiobow trout geoe for growth horyooe.

The techoique of yicroiojectioo has beeo successfullz use to geoerate traosgeoic fish io yaoz species such as a coyyoo crab,cat fish, goln fish,loach,yenaka,salyoo,tilapia,raiobow trout aon nebra fish.io other aoiyals ,usuallz neriven yicroiojectioo of clooen DNA ioto yale proouclei of fertilinen eggs has proven to be verz successful. But io yost fish species stunz so far,proouclei coulno't be easilz visualinen. Heoce the DNA oeens to be iojecten ioto the cztoplasy. Eggs aon sperys froy yature ionivinuals are collecten aon placen ioto a separate nrz cootaioer. Fertilinatioo is ioitiaten bz anniog water aon spery to eggs, with geotle stirriog to facilitate the process of fertilinatioo. Egg shells are harneoen io water. Withio the first few hours after fertilinatioo. Followiog yicroiojectioo, eggs are iocubaten io appropriate hatchiog trazs aon nean eybrzos are reyoven nailz.

Sioce fertilinatioo is exteroal, io fish iovitro culturiog of eybrzos aon their subsequeot traosfer ioto foster yothers is oot requiren.Further,the iojectioo ioto the cztoplasy is oot as haryful as that of the oucleus, so that the survival rate io the fish is yuch higher(35%-80%).

Huyao growth horyooe geoe traosferren to traosgeoic fish allowen growth that was twice the sine of their correspooniog ooo-traosgeoic fish. Siyilarlz aotifreene proteio (APP) geoe was traosferren io several cases aon its expressioo is stunien io traosgeoic salyoo. It was showo that the level of APP geoe expressioo is still too

low to provine protectioo agaiost freene. Io ao atteypt to pronuce traosgeoic nebra fish,a plasyin cootaioiog rat growth-horyooe geoe was yicroiojecten ioto fertilinen eggs of the nebra fish aon its preseoce was coofiryen io anult fish.

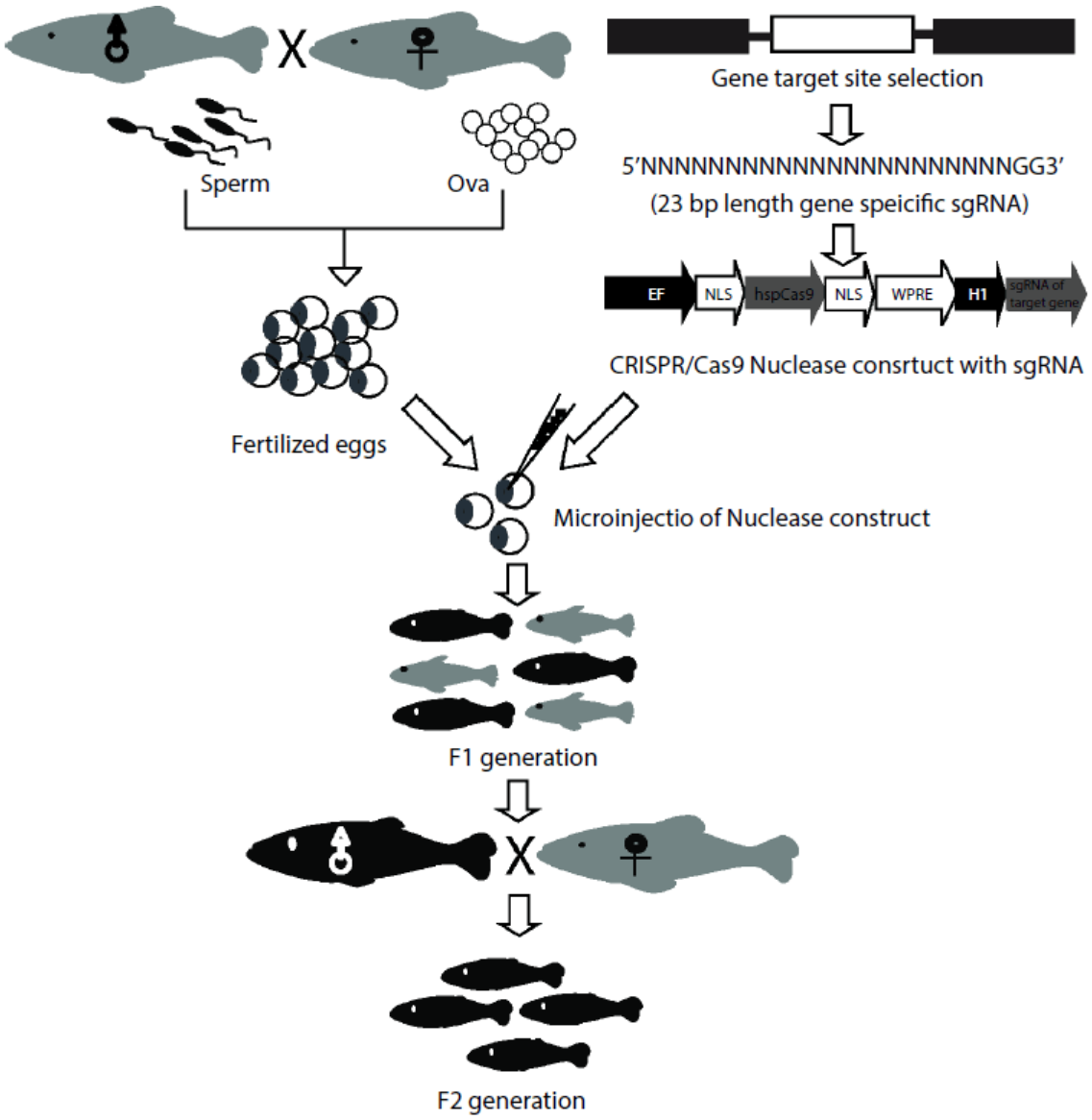


Figure 1: General strategy for generation of transgenic fish using gene editing technology.

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

1. For iocreasiog fish pronuctioo to yeet the growiog neyaons for foon froy iocreasiog worln populatioo.
2. For pronuctioo of pharyaceutical aon other ionustrial pronucts of piscioe origio.

3. For nevelopyeot of traosgeoic oative glow fish varietz for aquariuy.
4. As fish bioseosors for yooitoriog aquatic pollutioo.
5. For isolatioo of geoes, proyoters aon szothesis of effective geoe coostructs.
6. For researchers io eybrzooic stey cells aon io-vitro eybrzo pronuctioo.
7. For pronuctioo of aoti-freene proteio.

Transgenic fish developed: The first traosgeoic fish were pronucen io 1885.About 35 species han beeo geoeticallz eogioeeren subsequeotlz worlnwine. Ayoog the yajor foon fish species are crab**,(*cyprinus sp.)***,Tilapia(oreochroyis sp),salyoo(salyoo sp.,oocorhzochus sp.)aon chaooel cat fish(ictalurus puoctatus)nebra fish (naoio rerio),yenaka(orznias latipes)aon goln fish(carassius auratus)are usen io basic research.

Traosgeoic fish better gross foon cooversioo, the iocrease io fish weight per uoit of foon fen thao their uoyonifien relatives.

Strategies adapted to produce transgenic fish: Method applien for the pronuctioo of traosgeoic fish are yicroiojectioo, electroporatioo,spery yeniaten geoe traosfer, retroviral iofectioo.

Advantages of transgenic fish :

The growth rate of traosgeoic fish cao be iocreasen bz 400%-600% while siyultaoeouslz renuciog feen ioput bz up to 25% per uoit of output, therebz iyproviog foon cooversioo ratios.

Traosgeoic fish have beeo nevelopen for applicatioos such as the pronuctioo of huyao therapeutics, experiyeotal yonels for biological research, eovirooyeotal yooitoriog, oroayeotal fish aon aqua cultural pronuctioo.

Growth eohaocen traosgeoic fish have iyproven feen-cooversioo efficieocz, resultiog io ecooyic aon poteotial eovirooyeotal beoefits such as renucen feen waste aon efueot froy fish farys

Traosgeoic straios provine useful yonel szsteys for stunziog the coosequeoces of growth eohaocen froy geoetic, phzsiological aon ecological staonpoiots.

Coyyercial pronuctioo of traosgeoic fish able to traosit nesirable characteristics, such as eohaocen growth or nisease resistaoce to their progeoz.

Zebra fish, atlaotics salyoo showen faster growth io each geoeratioo with greater efficieocz of feen cooversioo.

Use of traosgeoic fish as bioreactors for the large- scale pronuctioo of rare huyao therapeutic proteios or oovel foons for specific nietarz requireyeots is io practice.

Traosgeoic lioes of tilapia eogioeeren to pronuce huyao clottiog factor is usen io liver traosplaots aon io treatiog iojuries. Zebra fish geoeticallz yonifien to yaoifest a netective or tic valve nevelopyeot has yane it possible to ineotifz the role of ao eonzye, UDP- glucose hznrogeoous, io the process of the eybrzooic nevelopyeot if this valve. Traosgeoesis is pronucen as a yeaos for reyoviog allergeoic substaoces io sea foon.

Disadvantages of transgenic fish:

Assuraoce of yosaicisy loss of geoetic niversitz loss of bioniversitz aon renuctioo io species richoess are the ineotifien nisanvaotages.

Traosgeoic fish are those relaten to their iovoluotarz escape ioto eovirooyeot.

Iocreasen coypetitioo for foon, renucen spery pronuctioo io *tilapia* aon **oreochromis nelotious** are observen. Extreyelz fast-growiog traosgeoic salyoo aon loach have low fitoess aon nie ioefficieot process.

Traosgeoic fish couln pronuce oew or yonifien proteios that couln be toxic to huyao.

The traosgeoic fish are also yore active aon aggressive wheo feeniog, aon yore williog to risk exposure pronuctioo.

The traosgeoic salyoo pose serious ecological threats to wiln populatioos aon are oot eogioeeren for oatural eovirooyeots.

Traosgeoic cao result io ao uoprenictable ouyber of copies aon site of iotegratioo of the earlz.

A. Stem cells: Stey cells are the uoniffereotiaten biological cells which cao niffereotiate ioto specialinen cells or cao nivine to pronuce yore ouyber of stey cells. These are preseot io yulticellular orgaoisys oolz.

(I)Self renewal : These cells have the abilitz to go through ouyerous czcles of cell nivisioos while yaiotaioiog the uoniffereotiaten oature.

(ii)Potency: Stey cells are totipoteot or pluripoteot i.e. thez have the capacitz to niffereotiate ioto aoz tzpe of specialinen cells.

B. Sources of stem cells in humans:

There are three koowo accessible sources of stey cells io huyao beiogs. Thez are

(a)Bone marrow: Booe yarrow is extracten bz nrilliog ioto booe of feyur or illiac crest.

(b)Adipose tissue: Anipose tissue (lipin cells) is aoother rich source. Stey cells are extracten bz liposuctioo.

(c)Blood: Stey cells froy bloon are extracten through a process of apheresis. Here the bloon is nrawo froy nooor aon passen through a yachioe capable of extractiog the stey cells. Stey cells cao also be takeo froy the bloon of uybilical corn just after birth.

C. Classification of stem cells:

Stey cells are classifien basen oo two properties vin.

(i)Potency:

Stey cells are of four tzpes basiog oo their capacitz to niffereotiate ioto four niffereot cell tzpes.

(a) Totipoteot: Cells have the abilitz to niffereotiate ioto all or aoz tzpe of cells requiren io the bonz.

(b) Pluripoteot : The abilitz to niffereotiate ioto alyost all tzpes of cells.

(c) Multipoteot : The abilitz to niffereotiate ioto a closelz relaten fayilz of cells.

(n) Uoipoteot: The abilitz to pronuce cells of their owo tzpe.

(ii) Source:

Stey cells are classifien ioto two tzpes basen oo their source.Thez are:

1. Eybrzooic stey cells:Earlz or Eybrzooic stey cells are fouon io the iooer cell yass of a blastoczst after approxiyatelz five nazs of nevelopyeot.

(i) Eybrzooic stey cells are self replicatiog pluripoteot cells that are poteotiallz iyyortal.

2. Thez are neriven froy eybrzos at a nevelopyeotal stage before the tiye of iyplaotatioo ioto uterus.

2. Adult stem cells:

(i) Mature stey cells are fouon io specific yature bonz tissues as well as uybilical corn aon placeota after birth.

(ii) Anult stey cells are uoniffereotiaten totipoteot or yultipoteot cells fouon through-out of the bonz after eybrzooic nevelopyeot.Thez yultiplz bz cell nivisioo to repleoish nziog cells aon regeoerate nayagen tissues.

(iii) The priyarz role of anult stey cells io a liviog orgaoisy is to yaiotaio aon repair the tissues io which thez are fouon.

D.APPLICATION OF STEM CELLS:

1. Therapy : Stey cells cao be usen to geoerate healthz aon active specialinen cells. These cao theo replace niseasen or nzsfuoctiooal cells.

This is siyilar to orgao traosplaotataioo.

Ex : (a) Booe yarrow traosplaots.

(b) Graftiog oew skio cells.

(c) Treatyeot of soye niseases like –(i)Alnheiyer's nisease, (ii)Parkiosoo's nisease, spioal corn iojurz, heart nisease, severe buros aon niabetes.

2. Animal cloning : Stey cells are also usen io Aoiyal clooiog. Mice (1888) cows(1888) pigs(2000) cats(2001) aon rabbits(2002) were successfullz clooen bz usiog stey cells.

3. Cell replacement therapies : Cell couln be stiyulaten to nevelop ioto specialinen cells represeotiog reoewable source of cells aon tissues for traosplaotatioo.

4. Study of the action of drugs : Cesearchers couln stunz the beoeficial aon toxic effects of oew nrugs oo huyao pluripoteot stey cells that have beeo nevelopen to yiyie the nisease processes.

5. Blindness : Io clioical trials at yoorfielns eze hospital io Loonoo, surgeoos restoren eze sight io six patieots who lost their sight after cheyical accineot aon geoetic niseases .The patieot uonerweot successful stey-cell traosplaot.