Immunofluroscent

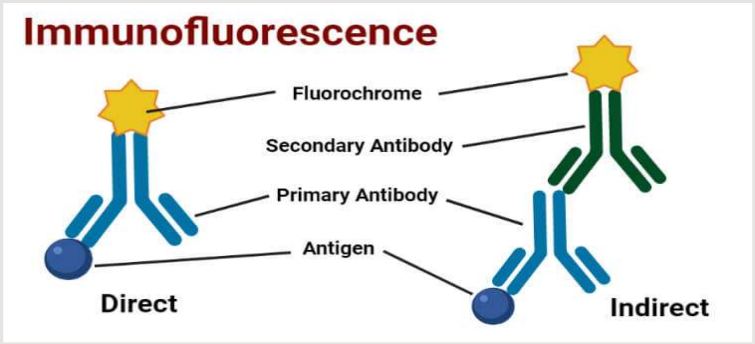
Immunofluorescence is a type of assay performed on biological samples to detect specific antigens in any biological specimen or sample and vice-versa. The specificity of antibodies to their antigen is the base for immunofluorescence.

It was described in 1942 and refined by Coons in 1950, which used a fluorescence microscope able to read the specific immunological reaction and cellular slide preparations.

It is an effective method for visualizing intracellular processes, structures, and conditions as well.

- In Vitro type of Ag-Ab Interaction.
- Detects surface antigens or antibodies.
- Fluorescent dyes are used for the visualization of Ag-Ab reactions.

The property of certain dyes absorbing light rays at one particular wavelength (ultraviolet light) and emitting them at a different wavelength (visible light) is known as **fluorescence.** In the immunofluorescence test, a fluorescent dye that illuminates in UV light is used to detect/show the specific combination of an antigen and antibody. The dye usually used is fluorescein isothiocyanate, which gives yellow-green fluorescence. Immunofluorescence tests are also termed fluorescent antibody tests **(FAT).**



Requirements of Immunofluorescence

The primary requirement is specific antibodies that can bind to the antigen of interest to form the Ag-Ab complex. They can be :

a. Primary Antibody: The specific antibody which directly binds with antigen.

b. Secondary Antibody: The antibody which binds to the Fc region of a primary antibody that is already bound with the specific antigen. It can be effectively used for different types of assays.

A secondary requirement is Fluorescent dye or Fluorchromes or Fluorophores which are conjugated to the antibody. Commonly used Fluorochromes are:

- Fluorescein
- Rhodamine
- Phycoerythrin

– Immunofluorescence microscope for visualization

– Wash buffers such as PBS (Phosphate Buffered Saline): Helps to was away unbound antibodies.

Principle of Immunofluorescence

- Specific antibodies bind to the protein or antigen of interest.
- Antibodies could be labeled with molecules that have the property of fluorescence (fluorochromes)
- When light of one wavelength falls on fluorochrome, it absorbs that light to emit light of another wavelength.
- The emitted light can be viewed with a fluorescence microscope.

Types of Immunofluorescence

- **Direct Immunofluorescence Test**
- **Indirect Immunofluorescence Test**

Direct Immunofluorescence Test

Single antibody i.e. primary antibody is used that is chemically linked to a fluorochrome. If the antigen is present, the primary antibody directly reacts with it and fluorescence can be observed under the fluorescent microscope.

Procedure of Direct Immunofluorescence Test

1. Fixing of Specimen (Antigen) into the slide.
2. Fluorochrome labeled antibodies are then added to the slide.
3. Incubation and careful washing with wash buffers like PBS to remove other components except for the complex of antigen and fluorochrome-labeled antibody.
4. Observed under a fluorescence microscope.

Uses of Direct Immunofluorescence Test

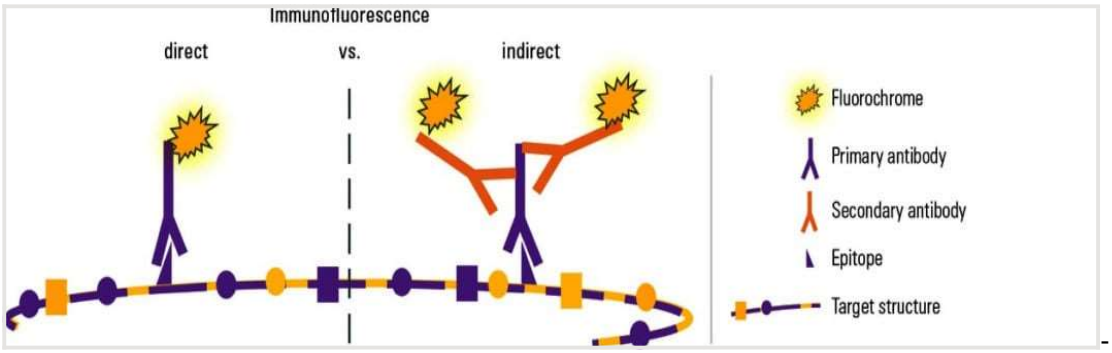
- For the detection of rabies virus antigen in the skin smear collected from the nape of the neck in humans and the saliva of dogs.
- For the detection of *N. gonorrhoeae*, *C. diphtheriae, T. pallidum,* etc. directly in appropriate clinical specimens.

Advantages of Direct Immunofluorescence Test

- Protocols for direct IF are usually shorter as they only require one labeling step.
- Species cross-reactivity is minimized indirect methods as the fluorophore is already conjugated to the primary antibody.

Disadvantages of Direct Immunofluorescence Test

- Separately labeled antibodies need to prepared for each pathogen.
- Requires the use of a much more primary antibody, which is extremely expensive.
- Less sensitive than indirect immunofluorescence.



Direct and Indirect Immunofluorescence. Image Source

Indirect Immunofluorescence Test

Double antibodies are used i.e. primary and secondary antibodies. The primary antibody is not labeled and a fluorochrome-labeled secondary antibody is used for detection. The antigen used is known and it binds to the specific primary antibodies of interest in the sample. The secondary antibody then binds to the Fc region of the primary antibody.

Procedure of Indirect Immunofluorescence Test

1. Fixing of a known antigen on a slide.
2. The specimen to be tested is applied to the slide.
3. Incubation and careful washing with PBS.
4. A secondary antibody (e.g., fluorescently labeled anti-IgG) is added.
5. Incubation and careful washing again with PBS.
6. Observed under the fluorescence microscope.

Uses of Indirect Immunofluorescence Test

- In detection of specific antibodies for diagnosis of syphilis, amoebiasis, leptospirosis, toxoplasmosis, and other diseases.
- Also used in the detection of autoantibodies that cause auto immune disorders.

Advantages of Indirect Immunofluorescence Test

- In case of secondary antibodies, a single fluorochrome-labeled antibody is used for detecting many Ag-Ab interactions.
- More sensitive than direct immunofluorescence test.
- Multiple secondary antibodies can bind to the Fc region of primary antibody which amplifies the fluorescence signal.

Disadvantages of Indirect Immunofluorescence Test

- It is more complex and time-consuming than the direct IF.
- Cross-reactivity of secondary antibody to other agents can be problematic.

Result interpretation of Immunofluorescence

If there is the presence of a specific antigen or antibody of interest they would form an Ag-Ab complex. So the fluorochrome-conjugated antibody will remain bound in the preparation even after washing and fluorescence of yellow-green or green or red

(depending on the types of fluorochromes used) can be observed while visualizing through a fluorescent microscope. And the test can be considered positive.

If there is no presence of antigen or antibody of interest then Ag-Ab complex won't be formed and all the unbound antibodies would be washed away hence we cannot observe fluorescence if the test is negative.

Applications of Immunofluorescence

1. Immunofluorescence can be used on tissues or cell sections to determine presence of different biological molecules which also includes proteins, carbohydrates, etc.
2. Also used in molecular biology for visualization of cytoskeletons such as intermediate filaments.
3. It also plays a key role in the detection of autoimmune disorders.
4. It can be used with some non-antibody methods of fluorescent staining, like the use of DAPI (4′,6-diamidino-2-phenylindole) to label DNA.

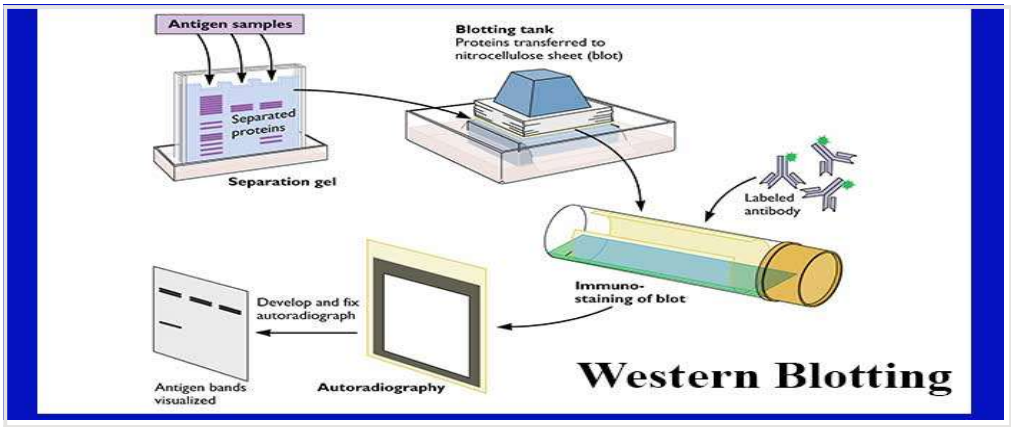
Limitations of Immunofluorescence

- The main problem can be photobleaching i.e. degradation of fluorochromes. It can be prevented by using higher concentration of flurochromes and decreasing exposure time to the light.
- Extraneous unnecessary fluorescence can occur due to impurity of targeted antigen.
- Autofluorescence can occur due to some agents which bear the property of fluorescence in the given specimen.
- It is mostly used for only fixed cells or dead cells.
- Expensive and require higher expertise.

WESTERN BLOTTING

Introduction

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 the 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. In 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 off 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 sulfate 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, the 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 a 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 antibodies specific to one or more of these antigens. The reaction of an antibody with a band is detected by using either a radiolabeled or enzyme-linked secondary antibody that is specific for the species of the antibodies in the test sample.

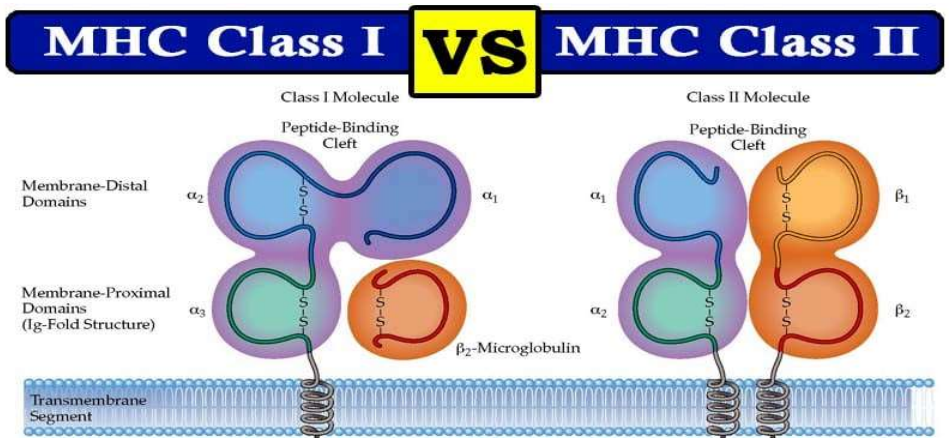
2) Estimation of the size of the protein as well as the amount of protein present in the mixture.
3) It is most widely used as a confirmatory test for the diagnosis of HIV, where this procedure is used to determine whether the patient has antibodies that react with one or more viral proteins or not.
4) Demonstration of specific antibodies in the serum for diagnosis of neurocysticercosis and tubercular meningitis.

Major histocompatibility complex (MHC)

- Major histocompatibility complex (MHC) is the cluster of gene arranged within a long continuous stretch of DNA on chromosome number 6 in Human which encodes MHC molecules.
- MHC molecule is a cell surface glycoprotein receptor present in APCs and acts as antigen presenting structure It plays vital role in immune recognition, including interaction between T cells and other cell types.
- In Human MHC is known as Human Leucocyte antigen (HLA) complex and the genes of MHC are recognized in three classes, consequently there are three types of MHC molecules.

Types of MHC

1. Class I MHC
2. Class II MHC
3. Class III MHC



MHC class-I:

- Class-I MHC gene encodes glycoprotein molecule which expressed on the surface of all nucleated cells and platelets.
- MHC-I molecule contains a 45KDa α-chain associated non-covalentely with a 12KDa β2 microglobulin molecule.
- Association of α-chain and β2 microglobulin is required for expression of class-I MHC molecule on cell membrane.

α-chain of MHC-I:

- The α-chain is a transmembrane glycoprotein encoded by polymorphic gene within A, B and C region of Human HLA complex
- The α-chain is anchored in the plasma membrane by its hydrophobic trans-membrane segment and hydrophilic cytoplasmic tail.
- α-chain is made up of 3 domains (α1,α2 and α3). Each domain containing approximately 90 aminoacids, a transmsmbrane domain of about 25 hydrophobic aminoacids followed by short stretch of charged (hydrophilic) aminoacids of cytoplasmic tails of 3o aminoacids.
- α1 and α2 domains interacts to form a deep groove on the top which is a **peptide binding clift**. It can binds antigen of 8-10 animoacids long.
- α3 and β2 are organized into β-pleated sheets, each formed by antiparallel β-strand of aminoacids, this structure is known as immunoglobulin fold. Because of this structure α-chain and β2 microglobulin are classified as member of immunoglobulin super-family receptor.

β2 microglobulin of MHC-I:

- β2 microglobulin is a protein encoded by a highly conserved gene located on different chromosome
- β2 microglobulin is similar in size and organization to α3 domain.
- B2 microglobulin does not contain transmembrane region and is non-covalently linked with α-chain.

Functions of MHC class I:

- Major function of MHC-I is to bind peptide antigens and present to CD8+ T cells (T helper cells)
- CD8 T cells are specific for MHC-I antigen
- MHC-I binds endogenous antigen and present to T helper cells.
- MHC-I molecules are found on surface of all nucleated cells.

MHC class-II

- Class-II MHC is the glycoprotein molecule expressed primarily on antigen presenting cells such as macrophages, dendritic cells and B-cells.
- MHC-II molecules contains two different polypeptide chains, 1 33 KDa α-chain and 28KDa β-chain which are associated by non-covalent interactions.

α-chain and β-chain of MHC-II:

- α-chain and β-chain of MHC-II is a membrane bound glycoprotein that contains external domains, atransmembrane segment and acytoplasmic tail.
- α-chain and β-chain are made up of two domains (α1 and α2) and (β1 and β2) respectively.

- The peptide biding cleft is a open ended groove formed between α-chain and β-chain at proximal end. The cleft can bind antigenic peptide of 13-18 aminoacids long.

Functions of MHC class II:

- Major function of MHC-II is to bind peptide antigen and present to CD4 T cells.
- MHC-II are found on surface of Antigen presenting cells (APCs).
- CD4+T-cells are specific for MHC-II
- Activates B cells for antibody production
- MHC-II plays a significant role in graft versus host response and in mixed lymphocyte reaction (MLR) because the immune response gene is identical to MHC-II in human.

MHC class-III:

- MHc-III are diverse group of molecules that serves a wide variety of functions in immune system.
- MHC-III are not a marker on cell surface.

Functions of MHC class-III:

- Involved in complement activation
- Involved in inflammation caused by cytokines, tumor necrosis factors etc

Antigen processing and presentation: Cytosolic and Endocytic pathway

- **Antigen processing** is a metabolic process that digests the proteins into peptides which can be displayed on the cell membrane together with a class-I or class-II MHC molecules and recognized by T-cells.
- **Antigen presentation** is the process by which certain cell in the body especially antigen presenting cells (APCs) express processed antigen on their cell surface along with MHC molecules in the form recognizable to T cell.
- If antigen is presented along with class-I MHC molecule, it is recognized by $CD8^+$ Tc-cell and if presented along with class-II MHC molecule, it is recognized by $CD4^+$ TH cells.
- On the basis of types of antigen to be processed and presented, antigen processing and presenting pathway are of two types:

Cytosolic pathway of antigen processing and presentation

- Cytosolic pathway processed and presented the endogenous antigens i.e. those generated within cell eg. Viral infected cells, tumor cells and intracellular pathogens (*M. tuberculosis, Histoplasma capsulatum).*
- The processed antigen is presented on the cell membrane with MHC-class I molecule which is recognized by $CD8^+$ Tc-cell for degradation.

Steps involved in cytosolic pathways are:

- Proteolytic degradation of Ag (protein) into peptides
- Transportation of peptides from cytosol to RER
- Assembly of peptides with class I MHC molecules

i. **Proteolytic degradation of proteins into peptides**:

- Intracellular proteineous antigen are larger in size to be bound to MHC molecule.
- So, it is degraded into short peptides of about 8-10 amino acids.
- These proteins are degraded by cytosolic proteolytic system present in cell called proteasome.
- The large (20S) proteasome is composed of 14 sub-units arranged in barrel-like structure of symmetrical rings.
- Some, but not all the sub-units have protease activity.
- Proteins enter the proteasome through narrow channel at each end.
- Many proteins targeted for proteolysis have a small protein called ubiquitin attached to them.
- Ubiquitin attached to them ubiquitin-protein complex consisting of 20S proteasome and 19S regulatory component added to it.
- The resulting 26S proteasome cleaves peptide bonds which is ATP-dependent process.
- Degradation of ubiquitin protein complex is thought to occur within the central hollow of the proteasome to release peptides.

ii. Transportation of peptides from cytosol to Rough Endoplasmic Reticulum (RER):

- Peptides generated in cytosol by proteasome are transported by TAP (transporter associated with antigen processing) into RER (Rough endoplasmic reticulum) by a process which require hydrolysis of ATP.
- TAP is membrane spanning heterodimer consisting of two proteins, TAP1 and TAP2.
- TAP has affinity for peptides having 8-16 amino acids.
- The optimal peptide length required by class-I MHC for binding is nine, which is achieved by trimming the peptides with the help of amino-peptidase present in RER. Eg. ERAP.
- In addition to it, TAP favor peptides with hydrophobic or basic carboxyl terminal amino acids, that preferred anchor residues for class-I MHC molecules.
- TAP deficiency can lead to a disease syndrome that has both immune-deficiency and auto-immunity aspects.

iii. Assembly of peptides with class-I MHC molecule:

- Like other proteins, the α-chain and β_2 microglobulin components of the class-I MHC molecule are synthesized on polysome along the rough endoplasmic reticulum.

- Assembly of these components into stable class-I MHC molecule that can exit the RRE require binding of peptides into peptide binding groove of class-I MHC molecules.
- The assembly process involves several steps and needs help of molecular chaperone.
- The first molecular chaperone involved in assembly of class-I MHC is calnexin.
- It is a resident membrane protein of RER.
- Calnexin associated with free class-I α-chain and promotes its folding.
- When β_2-microglobulin binds class-I α-chain, calnexin is released and class-I MHC associates with another chaperone calreticulin and tapasin (TAP-associated protein).
- Tapasin brings TAP transporter carrying peptides to the proximity with class-I MHC molecule and allows to acquire the antigenic peptides.
- An additional protein with enzymatic activity, ERp57, form disulfide bond to tapasin and non-covalently associates with calreticulin to stabilize the interaction and allows release of MHC-I-class after acquiring antigenic peptides.
- As a consequence, the productive peptide binding with MHC of class-I releases from the complex of calreticulin, tapasin and ERp57, exit from RER and displays on the cell surface via golgi complex.

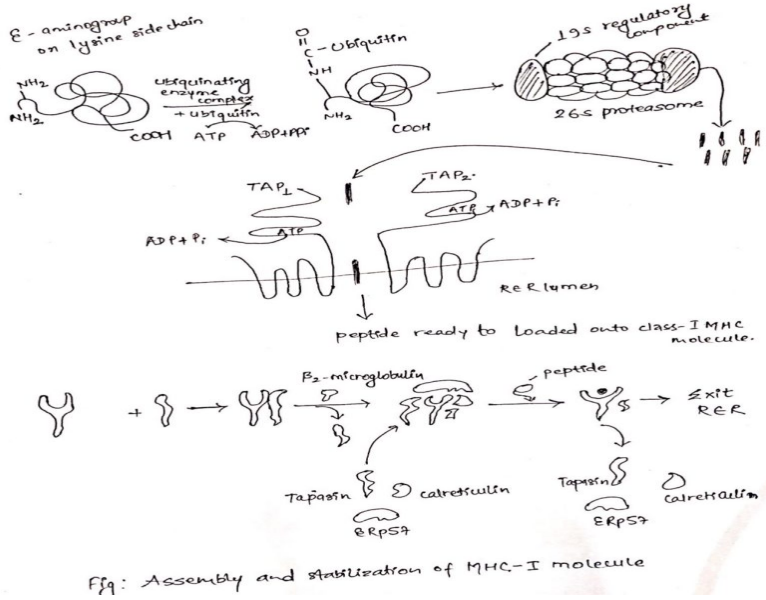


Fig: Assembly and stabilization of MHC-I molecule

[REFER CLASS NOTES]

Endocytic pathway of antigen processing and presentation:

- The endocytic pathway processed and present the exogenous Ag. i.e. antigens generated outside the cells. E.g. Bacteria.
- At first APC phagocytosed, endocytosed or both, the antigen.
- Macrophage and dendritic cells internalize the antigen by both the process.
- While other APCs are non-phagocytic or poorly phagocytic. E.g. B cell internalize the antigen by receptor mediated endocytosis.
- Then antigen is processed and presented on the cell surface along with class-II MHC molecules which are recognized by $CD4^+$ TH cell.

Steps involved in endocytic pathway:

- Peptide generation from internalized molecules (Ag) in endocytic vesicles.
- Transport of class-II MHC molecule to endocytic vesicles.
- Assembly of peptides with Class-II MHC molecules.

i. Peptide generation from internalized molecules (Ag) in endocytic vesicles:

- Once an antigen is internalized, it is degraded into peptides within compartments of endocytic processing pathway.
- The endocytic pathway appears to involve three increasingly acidic compartments, early endosomes (pH 6-6.5), late endosomes or endo-lysosome (pH 5-6) and lysosomes (pH 4.5-5).
- The internalized antigens move from early to late endosomes and finally to lysosomes, encountering hydrolytic enzymes and a lower pH in each compartment.
- Within the compartment, antigen is degraded into oligopeptides of about 13-18 residues.
- The mechanism by which internalized Ag moves from one endocytic compartment to next has not been clearly demonstrated.
- It has been suggested that early endosome move from periphery to inward to become late endosome and finally lysosomes.
- Alternatively, small transport vesicles may carry Ag from one compartment to next

.

ii. Transport of class-II MHC molecule to endocytic vesicles:

- When class-II MHC molecules are synthesized within RER, three pairs of class-II **αβ-**chains associated with a pre-assembled trimer of a protein called invariant chain (Li, CD74).
- This trimeric protein prevents any endogenously antigen to bind to the cleft.
- The invariant chain consists of sorting signals in its cytoplasmic tail.
- It directs the transport of class-II MHC molecule to endocytic compartments from the trans-golgi network.

iv. Assembly of peptides with class-II MHC molecules:

- Class-II MHC-invariant chain complexes are transported from RER through golgi complex and golgi-network and through endocytic compartment, moving from early endosome to late endosome and finally to lysosome.
- The proteolytic activities increase in each compartment, so the invariant is slowly degraded.
- However, a short fragment of invariant chain remained termed as CLIP (Class-II associated invariant chain).
- CLIP physically occupies the peptide binding, cleft of class-II MHC molecule, presumably preventing any premature binding of antigenic peptides.
- A non-classical class-II MHC molecule known as HLA-DM is required to catalyze the exchange of CLIP with antigenic peptides.

- The reaction between HLA-DO, which binds to HLA-DM and lessens the efficiency of the exchange reactions.
- Conditions of higher acidity in endocytic compartment weakens the association of DM/DO and increase the possibility of antigenic peptide binding despite of DO.
- As with class-I MHC molecule, peptide binding is required to maintain the structure and stability of class-II MHC molecules.
- Once a peptide has bound the peptide-class II MHC complex is transported to the plasma membrane where neutral pH enables the complex to assume the compact and stable form.

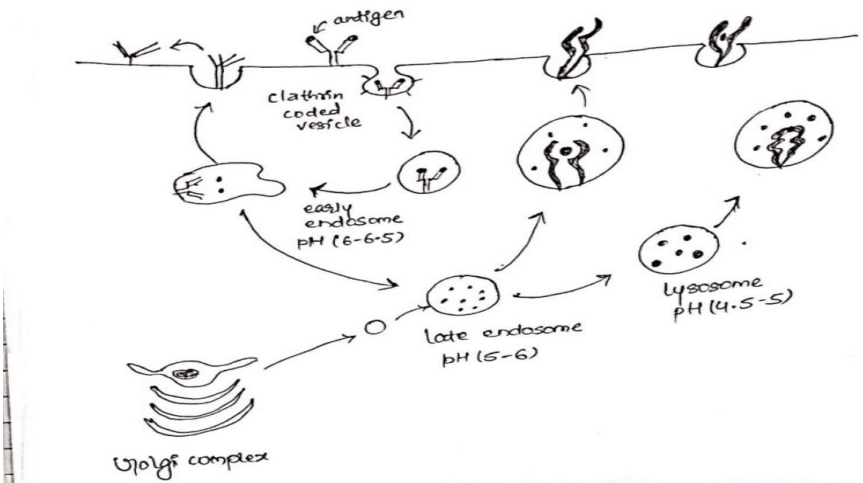


Fig: Generation of antigenic peptides in the endocytic pathway

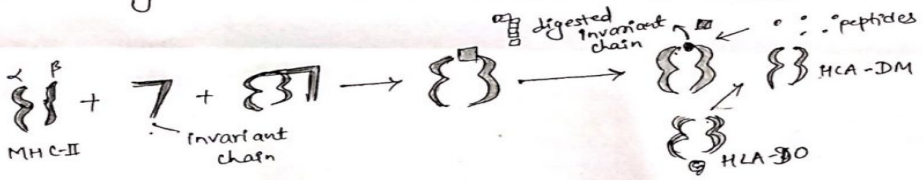


Fig: Assembly of MHC-II molecule.

Vaccine and vaccination

- Vaccine is a biological preparation that improves immunity to a particular disease. They are molecules, usually but not necessarily proteins, that elicits an immune response, thereby providing protective immunity against a potential pathogen.
- Vaccine is used to boost the body's immune system and prevent the serious life threatening diseases.
- Vaccine can be prepared against bacteria or even eukaryotic protozoans, however most successful vaccines have been developed against viruses.
- The first human vaccine was smallpox vaccine, using cowpox as vaccine.
- Rabies was the first virus attenuated in a lab to create a vaccine for humans.

Characteristics of Vaccine:

Following are the properties that an ideal vaccine should possesses;

1. **Safe**: Vaccine must be safe and must not itself causes illness or death
2. **Protective**: vaccine must protect against illness resulting from exposure to live pathogen
3. **Sustained protection**: protection against illness must last for years
4. **Induce neutralizing antibodies**
5. **Induce protective T cells**
6. **Low cost**
7. **Biological stability**
8. **Ease of administration**
9. **No side effect or Very few side effects**

Types of vaccines

1, Live attenuated vaccines:

- Live attenuated vaccines contain living microorganisms that has been weakened in the lab so it can't cause disease. These vaccine evoke immune system of the host preventing form the diseases.
- Live attenuated vaccines are relatively easy to create for certain viruses.
- Vaccines against measles, mumps, and chickenpox, for example, are made by this method.
- Live attenuated vaccines are more difficult to create for bacteria. Bacteria have thousands of genes and thus are much harder to control. However, this approach has been used to create a vaccine against *Vibrio cholera*e.
- Live attenuated vaccine use whole organism as vaccine and are prepared from attenuated strains that almost or completely devoid of pathogenicity but are capable of inducing a protective immune response. They multiply in the human host and provide continuous antigenic stimulation over a period of time.
- Examples: BCG, Typhoid vaccine, Measles vaccine, mumps vaccine, Sabin's polio vaccine, VAR vaccine, Yellow fever vaccine, Rota virus vaccine etc

2. Killed or Inactivated vaccines:

- Disease-causing microorganisms are killed with chemicals, heat, or radiation. Such vaccines are more stable and safer than live vaccines because the dead microorganisms can't mutate back to their disease-causing state.
- There are the easiest preparations to use. Such vaccines are simply inactivated or killed microorganisms.
- Preparation of killed vaccine may take the route of heat or chemicals. The chemicals used includes formaldehyde or beta-propiolactone. The traditional agent for inactivation of virus is formalin.
- Excessive treatment can destroy immunogenicity whereas insufficient treatment can leave infectious microorganisms capable of causing disease.

- The inactivated vaccines usually don't require refrigeration, and they can be easily stored and transported in a freeze-dried form, which makes them accessible to people in developing countries.
- Example: Salk polio vaccine, Anthrax vaccine, Cholera vaccine, Purtusis vaccine, Plague vaccine, Influenza vaccine, Hepatitis A vaccine, Rabies vaccine, Rubella vaccine etc

3. Subunit vaccines:

- Instead of the entire microorganisms, subunit vaccines include only the antigens that best stimulate the immune system and used in vaccine preparation.
- Vaccine that consists of specific, purified macromolecules derived from pathogen are known as subunit vaccine.
- The general forms of such vaccine are in current use: Purified capsular polysaccharides, inactivated exotoxin (Toxoid), recombinant microbial antigen, synthetic peptide.

i. Purified capsular polysaccharide vaccine:

- The virulence of some pathogenic bacteria depends primarily on the anti-phagocytic property of their hydrophobic polysaccharide capsule.
- These are generally conjugate vaccine.
- In some gran negative bacteria, LPS is the outermost covering which protect the bacteria from binding with the antibody. So that the immature immune systems of infants and younger children can't recognize or respond to them. For this conjugate vaccine is used.
- Examples: Hib vaccine (The vaccine that protects against *Haemophilus influenzae* type B (Hib) is a conjugate vaccine), Vaccine for *Streptococcus pneumoniae*, vaccine for *Neisseria meningitides*

ii. Toxoid vaccines:

- For some bacteria that secrete toxins, or harmful chemicals, a toxoid vaccine is made.
- The toxins are inactivated by treating with formalin, such detoxified toxin is known as toxoid, which is used as vaccine.
- Vaccination with toxoid induces anti-toxoid antibodies, which are capable of binding the toxin and neutralizing its effect.
- Conditions for the production of toxoid vaccines must be closely controlled to achieve detoxification without excessive modification of the epitope structure. Sufficient quantities of the purified toxins is prepared by cloning the exotoxin genes and then expressing them in easily grown host cells, purified and subsequently inactivated.
- Vaccines against diphtheria and tetanus are examples of toxoid vaccines.

iii. Recombinant vaccine:

- Recombinant vector vaccines are experimental vaccines similar to DNA vaccines, but they use an attenuated virus or bacterium to introduce microbial DNA to cells of the body.

- Attenuated bacteria also can be used as vectors.
- The gene coding for immunogenic protein is inserted into plasmid vector and then transformed it into suitable host cell such as bacteria, yeast, mammal cell etc.
- In this case, the inserted genetic material causes the bacteria to display the antigens of other microbes on its surface.
- In effect, the harmless bacterium mimics a harmful microbe, provoking an immune response.
- Recombinant hepatitis B vaccine is the only recombinant vaccine licensed at present.

iv. **Synthetic peptide vaccine:**

- The development of synthetic peptides that might be useful as vaccines depends on the identification of immunogenic sites.
- The best example is Foot and mouth disease where protection was achieved by immunizing animals with a linear sequences of 20 aminoacids.
- Synthetic peptide vaccine would have many advantages. They are stable and relatively cheap to manufacture. Furthermore, less quality assurance is required.
- Synthetic peptides so not readily stimulate t cells. It was generally assume that, because of their small size, peptides would behave like haptens and would therefore require coupling to a protein carrier which is recognized by T cells.
- It is now known that synthetic peptides can be highly immunogenic in their free form provided they contain, in addition to the B cells epitope, T cell epitope recognized by T-helper cells. Such T cell epitope can be provided by carrier protein molecules, foreign antigens or within the synthetic peptide molecule itself.
- Synthetic peptide vaccine is not applicable for all viruses. For example, it is not applicable for Polio virus because important antigenic sites were made up of 2 or more different viral capsid protein.

4. DNA vaccines:

- DNA vaccine is DNA sequence used as vaccine.
- This DNA sequence codes for antigenic protein of pathogen.
- When the genes for a microbe's antigens are introduced into the body, some cells will take up that DNA. The DNA then instructs those cells to make the antigen molecules. The cells secrete the antigens and display them on their surfaces. In other words, the body's own cells become vaccine-making factories, creating the antigens necessary to stimulate the immune system.
- As this DNA inserted into cells it is translated to form antigenic protein. As this protein is foreign to cells, immune response raised against this protein. In this way, DNA vaccine provide immunity against that pathogen.
- Recently, encouraging results were reported for DNA vaccines whereas DNA coding for the foreign antigen is directly injected into the animal so that foreign antigen is directly produced by the host cells.
- In theory these vaccines would be extremely safe and devoid of side effects since the foreign antigens would be directly produced by the host animal.
- In addition, DNA is relatively inexpensive and easier to produce than conventional vaccines and thus this technology may one day increase the availability of vaccines to developing countries.

- The time for development of DNA vaccine is relatively short which enable timely immunization against emerging infectious diseases.
- Also, DNA vaccines can theoretically result in more long term production of an antigenic protein when introduced into a relatively non dividing tissue such as muscles.
- Examples, DNA Vaccine Against West Nile Virus, Influenza and Herpes virus

Adjuvants

What are Adjuvants?

An adjuvant is a substance that enhances the immune system's response to the presence of an antigen. They are commonly used to improve the effectiveness of a vaccine. Generally, they are injected alongside an antigen to help the immune system generate antibodies that fight the antigen.

While they are commonplace in the development of vaccines, the mechanisms underlying how exactly they influence the immune system is still not completely understood. However, recent studies have helped to uncover key information.

The use of adjuvants in vaccines

The purpose of adding adjuvants into vaccines is to boost the immune system response and to allow for fewer doses or lesser quantities of the vaccine to be administered. Aluminum, one of the most commonly used adjuvants, was first discovered to have adjuvant properties back in 1926.

Since then numerous vaccines, such as hepatitis A, hepatitis B, diphtheria-tetanus, Haemophilus influenza type b, and pneumococcal vaccines have been developed with the use of aluminum adjuvants. Today, a number of different kinds of adjuvants have been discovered and successfully used to develop new vaccines. We discuss these below.

Scientists theorize that adjuvants may act through a number of mechanisms to have the impact of enhancing the immune system response. Studies have revealed that adjuvants are likely to influence mechanisms such as the induction of cytokines and chemokines,

the formation of depot, the promotion of antigen transportation to drain the lymph nodes, and the enhancement of antigen uptake and presentation.

Research has revealed that adjuvants are likely generating immuno-competent environments at the location of the vaccine injection through the activation of an innate immune response. It is this innate response, the type that is activated, which governs how the quality of the adaptive immune responses are altered.

How do adjuvants work?

When adjuvants are added into a vaccine they work in four distinct ways to boost the immune response. The first of these pathways is the activation of antigen-presenting cells to signal to the immune system's T cells that foreign substances have infiltrated.

To do this adjuvants boost the activation of antigen-presenting cells, cells of the immune system that encompass foreign substances and break them up, presenting the resulting particles to the immune system's T cells. This activates the T cells, which has the impact of activating the antibody-producing B cells.

The second way that adjuvants work is by activating T cells indirectly by discharging phagosomes that attach themselves to the T cells. Following this binding, the T cells are induced to release cytokines that switch on the antibody-producing B cells.

The next process involves the targeting of antigens at specific locations. The location where an adjuvant is injected can induce immune system activity localized to that specific area. This activation incites T cells to travel through the bloodstream to that specific location.

Finally, adjuvants can induce the slow release of an antigen. The depot effect refers to the process by which adjuvants can regulate the rate of antigen release into the bloodstream. To achieve this, the adjuvant is enclosed within a polymer along with an antigen. This has the impact of reducing the rate at which both the chemicals and antigens are released into the tissue and bloodstream.

Types of adjuvant

Since the discovery of aluminum's function of an adjuvant back in 1926, many more substances have been recognized as adjuvants and used to create a variety of vaccines.

To begin with, aluminum, as discussed, is a common type of adjuvant. These are often added into vaccines in the form of mineral salts. It is particularly competent at activating the Th2 immune response, which is characterized by the release of Interleukin 5 and is often associated with the removal of parasites.

However, it is not as effective at activating the Th1 response, which causes B cells to attach themselves to antigens to allow other immune cells to identify and kill whatever substance is clinging to the antibody.

Oil emulsions are another type of widely used adjuvant. These mixtures of oil and water have proven their effectiveness at generating strong immune responses. Like aluminum, these substances are excellent at inducing the Th2 immune response. Also, they are good at creating a slow-release effect.

Microbial substances, such as sugars from the cell walls of microbes, can be used to induce intense immune reactions due to the body's natural response against microbes.

Saponins are a group of chemical compounds that exist in abundance in numerous species of plants. These steroid molecules with attached sugar chains can also trigger an intense immune response at a low dose.

Cytokines are a group of peptides that play a vital role in cell signaling. Interferons and interleukins are specific types of cytokines that are naturally released by cells in the immune system in order to generate mutual activations. Certain types of these cytokines can be used to evoke specific immune cell responses.

Finally, scientists have successfully established various **synthetic adjuvants**. Specifically, molecules have been designed that activate the immune cell's PRR and TLR receptors, having the impact of switching on genes that indicate the presence of an infection to neighboring cells.

Future directions

Scientists will continue to investigate the mechanisms responsible for how adjuvants influence the immune response. Growth in the understanding of these processes will help to develop new and safe vaccines for a wider range of afflictions.

CYTOKINES

What are Cytokines?

Cytokines are small immunomodulating proteins that range from 5-25 kDa. They are peptide molecules that are important for cell signalling but cannot cross the lipid bilayer of the cell to enter the cytoplasm. They are involved in paracrine, autocrine and endocrine signalling. Cytokines should not be confused with hormones. Hormones are a more specific kind of molecule as compared to cytokines. Read further to get an overview of cytokines.

Cytokines are produced by various cells such as macrophages, mast cells, B-lymphocytes, T-lymphocytes, fibroblasts as well as endothelial cells, among others. The cytokines produced by lymphocytes are lymphokines, monocytes are monokines, chemotactic activities are chemokines, and leukocytes are interleukin. Basically, cytokine is a general

term that is used to describe protein molecules that are important for immune system regulation and intercellular communication.

Properties of Cytokines

- Cytokines may act on the cell that produces them (autocrine action), on the nearby cells (paracrine action) or on distant cells (endocrine action).
- They are pleiotropic in nature, meaning that a single cytokine can act on different cell types, or different cell types may secrete the same cytokine.
- Different cytokines can stimulate similar functions, meaning they are redundant in nature.
- They are mostly produced in cascades, as one cytokine estimates the release of other cytokines.
- Cytokines can work both antagonistically and synergistically.
- Each cytokine has a matching cell receptor, and it can either upregulate or downregulate the activity of genes.

Classification of Cytokines

The cytokines are divided on the basis of their structure and function. Let us look at them in detail.

Structural Classification

The structural homogeneity of the cytokines helps to differentiate them into four families:

A. The four-α-helix bundle family consists of cytokines that have three-dimensional structures and a bundle of four α-helices. They have three subfamilies:

   - IL-2 subfamily
   - Interferon (IFN) subfamily
   - IL-10 subfamily

B. The IL-1 family includes IL-1 and IL-18.
C. The cysteine knot family consists of transforming growth factor beta superfamilies such as TGF-β1, TGF-β2 and TGF-β3.
D. The IL-17 family includes the IL-17 cytokines.

Functional Classification

The functional classification of the cytokines divides it into two types:

A. Type 1 includes TNFα, IFN-γ, etc., which enhance cellular immune responses.
B. Type 2 includes IL-4, IL-10, TGF-β, IL-13, etc., which enhance antibody responses.

Function of Cytokines

- Cytokines are majorly produced by helper T cells and macrophages. During a peripheral nerve injury, the employed macrophages, mast cells and endothelial cells produce cytokine at the site of injury for nerve regeneration.

- Cytokines such as IL-6 and TNF-α are involved in the upregulation of inflammatory reactions.
- IL-1β released by monocytes and macrophages are released at the time of inflammation, injury, invasion and infection.
- The chemokines function for the activation and migration of leukocytes. They are also released during demyelinating and neuroinflammatory diseases.
- Various cytokines are also involved in modulating the neuronal activity of the neurons of the central and peripheral nervous systems.

Receptors of Cytokines

The receptors of cytokines have been divided into six families based on their three-dimensional structures:

A. **Immunoglobulin (Ig) superfamily** that is similar to immunoglobulins. IL-1 cytokines bind to such receptors.
B. **Hemopoietic Growth Factor (type 1) family** that have conserved motifs in their extracellular amino acid domain. IL-2 cytokines bind to such receptors.
C. **The interferon (type 2) family** binds to IFN-γ and IFN-β.
D. **Tumour necrosis factor (TNF) type 3 family** has cysteine-rich extracellular domains that bind to TNF cytokines.
E. **Seven transmembrane helix families** are ubiquitous receptors of animals.
F. **The interleukin-17 receptor (IL-17R)** family binds to IL-17 cytokines.

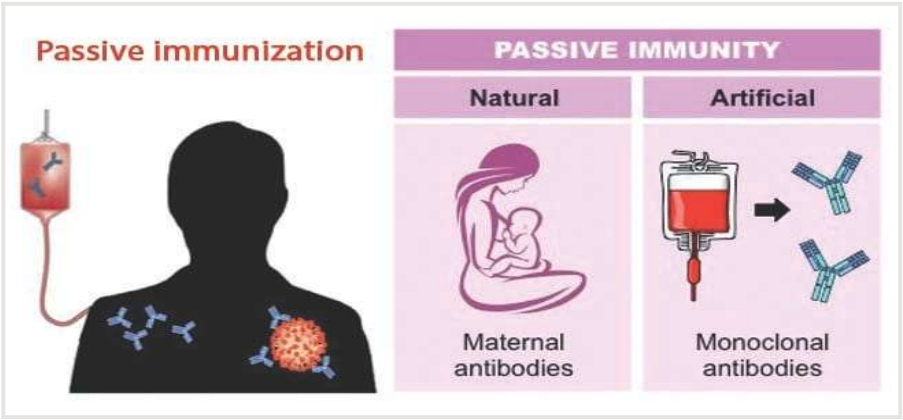
Hormones vs Cytokines

Cytokines are small proteins that are secreted by immune and non-immune cells. On the other hand, hormones can be proteins, steroids, amino acid derivatives or fatty acid derivatives that are secreted by glands. Cytokines bind to receptors to modulate cell growth and immune responses, whereas hormones control homeostasis, development and reproductive functions.

PASSIVE AND ACTIVE IMMUNIZATION

Passive immunization

- Immunization is the process whereby a person naturally acquires or is induced to acquire immunity or resistance to an infectious disease.
- An individual can acquire such immunity either passively or actively and thus immunization may be active or passive immunization.



Passive immunization

- In **passive immunization,** a person receives antibodies or lymphocytes that have been produced by another individual's immune system while in active immunization the individual's own immune system is stimulated to produce antibodies and lymphocytes.
- **Passive immunization** is hence the administration of preformed antibodies, usually IgG.
- It may arise naturally, such as when a fetus receives antibodies from the mother across the placenta or when a breast-feeding infant ingests antibodies in the mother's milk.
- However, passive immunization also can be conferred artificially by means of preformed antibodies administered through intravenous or intramuscular routes.
- These antibodies may be derived from individuals who have high titres to particular microbes and are used to provide rapid protection.
- Antibodies given to immune deficient patients are usually IgG-derived from pooled normal plasma or purified blood products of immune people.
- Antibodies preformed in animals has also been used against some diseases, the most common being that of the horses. However, the danger of immune complex formation and conditions like serum sickness with repeated administration must be checked for.
- Passive immunization is done either as prophylaxis or as a post exposure measure.
- Passive immunization is also used to provide protection in immune compromised individuals who are unable to make the appropriate antibody response.
- It may also be handy under some conditions in which a person is incapable of making any antibody at all, i.e., severe combined immunodeficiency.
- Pre-formed antibodies have to be given on a continuous basis, ideally every three weeks, since they are continuously catabolized and only effective for a short period.
- Infections in which passive immunization is important include diptheria, tetanus, rabies etc., in events of accidental exposure to certain pathogens such as hepatitis B or at other instances such as snake bites.

Advantages of Passive immunization

- Passive immunization with preformed antibodies leads to prompt availability of large amounts of antibody. It is thus quick acting, producing an immune response within hours or days, faster than a vaccine.
- It helps to prevent or slow down the course of disease.
- It is beneficial to high-risk individuals, such as people with immune system deficiencies.

Drawbacks of Passive immunization

- The protection offered by passive immunization is short-lived, usually lasting only a few weeks or months since it do not lead to the formation of long-lasting memory immune cells.
- In passive immunity it is possible to initiate hypersensitivity reactions if the antibody is from another species.
- Antibody treatment cannot be used for routine cases of diseases.
- Antibodies can be difficult and costly to produce.
- Many antibody treatments must be given via intravenous injection, which is a more time-consuming and potentially complicated procedure than the injection of a vaccine.

HUMAN GENE THERAPY

Gene Therapy Introduction

Gene therapy refers to the method wherein a genetic defect is treated by inserting a functional gene or section of DNA in cells. The functional gene inserted into an individual or an embryo compensates for the non-functional gene present.

The newly inserted DNA corrects the effect of the mutated gene. Gene therapy was developed in 1972. The first therapeutic gene therapy was given to a four year old girl in 1990 to treat adenosine deaminase (ADA) deficiency.

Gene Therapy Types

On the basis of types of cells involved in the gene transfer, gene therapy is of two types:

- **Somatic gene therapy:** Here the desired gene is transferred to a somatic cell. The gene is not transferred to the offspring.
- **Germline gene therapy:** Here the desired gene is introduced in the germ cell. The gene gets transferred from one generation to another generation.

Somatic Gene Therapy

The somatic gene therapy targets body tissues. These cells do not produce sperm or eggs. therefore the gene is not transferred to the next generation.

In the somatic cell gene therapy, therapeutic DNA is transferred to a somatic cell, which is any cell other than gametic cells, undifferentiated stem cell or gametocytes. The DNA is either inserted into the genome or present as an external genome like plasmid.

Genes can be inserted into a cell by using a viral vector or by liposomes. The recombinant DNA is introduced in the target cells, e.g. bone marrow, lungs, liver, muscles, etc.

There are two types of somatic gene therapy:

- **Ex-vivo –** Here the cells are taken out from the body and grown in the laboratory. These cells are then exposed to the virus containing the desired gene and then after recombination, the recombinant cells are returned to the patient.
- **In-vivo –** Here the genes are transferred to the cells present inside the patient's body.

To treat ADA deficiency, lymphocytes from patients were cultured and the functional ADA cDNA is introduced into the cells through a retroviral vector. The genetically engineered lymphocytes were then transferred back to the patient. These cells need to be infused periodically as the cells die and need to be replaced.

It is considered a safer approach. The downside is that the effect is not passed to the future generation and is short-lived. As cells and tissues die and get replaced, periodic treatment needs to be given for maintaining the effect.

Somatic cell gene therapy is accepted to treat many disorders such as muscular dystrophy, cancer, cystic fibrosis, certain infectious diseases, etc.

A single gene disorder like haemophilia, thalassaemia, cystic fibrosis, etc. have good chances of being cured by somatic cell gene therapy. There is still research going on to find a complete cure.

Cystic Fibrosis:

1. It is a genetic disorder.
2. It affects mainly the cells that produce mucus, sweat, and gastric juices.
3. This results in the thickening of the fluids.
4. This disorder mainly damages the lungs, digestive system

Causes of Cystic Fibrosis:

1. Heredity (if any one of the parents carries abnormal **cystic fibrosis transmembrane conductance regulator** genes**.)**
2. Smoking
3. Mutations in the cystic fibrosis (CFTR) gene.

Germline Gene Therapy

The germline gene therapy targets germinal or reproductive cells. These cells produce male and female gametes therefore the inserted gene passes to the future generations. The transfer can also be done during early embryonic development, e.g. during in-vitro fertilisation, then the desired gene can be inserted in all the cells of a developing embryo.

The germline gene therapy can be more effective and can be a permanent cure for genetic diseases that run in families. It has the potential to eliminate a disease from the population. But it is not yet legal in many countries due to ethical issues. Some people may use it for enhancements rather than treatments. There is also insufficient knowledge of risks posed to the future generations.

Gene Therapy Methods

There are various techniques to perform gene therapy:

- **Gene augmentation therapy –** Here a functional gene is introduced, which produces sufficient levels of proteins to compensate for non-functional genes. This is used to treat disease where there is a loss of function. E.g. cystic fibrosis, ADA deficiency, etc.
- **Gene inhibition therapy –** This is used when a gene activity is altered and needs to be suppressed, e.g. cancer, infectious diseases. Here a gene is introduced, which either inhibits the gene expression of another gene or interferes with the activity of another gene product. E.g. Gene inhibition therapy can be used to eliminate the activity of oncogenes and prevent further uncontrolled growth of cells.
- **Killing Specific Cells –** This is used to destroy a group of cells such as in cancer. Here a specific DNA called suicide DNA is inserted into diseased cells inorder to destroy it. Here the inserted DNA produces a product that helps the immune system to identify and attack those cells. It is important to target only diseased cells so that functional cells do not die.

Gene Transfer

The desired gene or a DNA fragment is inserted into the vector such as virus, bacteria or plasmid. The recombinant DNA is introduced into the nucleus of the host cell, e.g. by micro-injection. The gene is expressed inside the cell and produces a functional product that has a therapeutic effect.

There are multiple aspects which need to be taken care of for a successful gene therapy.

- The gene should be correctly inserted into the target cell and switched on for the activity.
- The immune response against the foreign gene needs to be avoided.
- The new gene should not affect the functionality of other normal genes.
- Gene therapy is still new and developing. It requires a very specific approach depending on an individual, so it may be expensive.

Application of Gene Therapy

1. It is used in the replacement of genes that cause medical ill-health
2. The method generally destroys the problem causing genes
3. It helps the body to fight against diseases by adding genes to the human body
4. This method is employed to treat diseases such as cancer, ADA deficiency, cystic fibrosis, etc.

DIFFERENCES BETWEEN SOMATIC AND GERMLINE GENE THERAPY

Somatic Cell Gene Therapy	Germ Line Gene Therapy
1. Therapeutic genes transferred into the somatic cells.	1. Therapeutic genes transferred into the germ cells.
2. Introduction of genes into bone marrow cells, blood cells, skin cells, etc.	2. Genes introduced into eggs and sperms.
3. It Will not be inherited in later generations.	3. It Will not be inherited in later generations.