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BT501

Health Biotechnology

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BT501 HEALTH BIOTECHNOLOGY

Full book Handouts (1-120)

Prepared By Mehwish Mughal

1-What is biotechnology all about?

Biotechnology

- bio - life Technology - the application, or harnessing of science for a specific purpose
- technology, process or practice - modifies or harnesses any living organism or system to be useful to any human purpose

Classical Biotech vs Modern

- Classical Biotech –since ages ,Growing better crops, Breeding animals
- Cheese and yogurt production – use of microorganisms
- Mendel's work – Laying foundation of modern Biotech
- Major discoveries : DNA structure, Ligase, Restriction enzyme
- Birth of rDNA technology
- Cut Gene from one organism and paste into others

Applications of Biotech

- AgriBiotech –: Pest-resistant, Herbicide resistant, Abiotic stress tolerant crops
- Environmental Biotech: Bioremediation, Bacteria and Fishes as Biosensors
- Health Biotech: biotherapeutics (insulin, growth hormones) Diagnostics tools (PCR, FISH, microarray)

The field of biotechnology has been in use for ages in various forms, which include the growing of better crops (agricultural biotechnology) and animal breeding (animal biotechnology). Similarly, the use of biotechnology has been around for thousands of years, especially the application of microorganisms in the production of cheese and yogurt (food biotechnology). In addition, the tools of biotechnology have been implied in animal husbandry, to develop pest-resistant crops, bioremediation (environmental biotechnology), as well as in bioethanol production. But the most promising application of biotechnology is found to be in the medical field by generations of biotherapeutics (insulin, growth hormones) and diagnostics tools (PCR, FISH, micro-array technique). Before we discuss various

applications of medical biotechnology, let us briefly go through the historical aspects of biotechnology and this information will make you to understand the field better.

It all began with the discovery made by Sir Alexander Fleming in the year 1918 where he observed that the mold *Penicillium* inhibited the growth of human skin disease-causing bacteria called *Staphylococcus aureus*. The discovery by Sir Alexander Fleming led to the making of antibiotics that we use today. These antibiotics are highly recommended and extensively used medicinally for bacterial infections. These antibiotics are basically substances produced by microorganisms that normally inhibit the growth of other microorganisms. Later on, antibiotics became widely available as a drug for treating microbial infections in human beings, especially with the development of penicillin as the most used antibiotic. Currently, a variety of microorganisms have been used to generate thousand liters of antibiotic drugs by using advanced biotechnology tools.

The field of biotechnology has taken a leap with the discovery of the double-helix structure of the deoxyribose nucleic acid (DNA) molecule and the credit goes to one research publication titled “This structure has novel features which are of considerable biological interest” authored by James Watson and Francis Crick in 1953, which claimed to discover the structure of the human DNA helix, the molecule that carries genetic information from one generation to the other. Nine years later, in the year 1962, they shared the Nobel Prize with Wilkins for cracking one of the most important of all biological puzzles. This discovery has led to the birth of genetic mapping, manipulation, and genetic engineering-type fields.

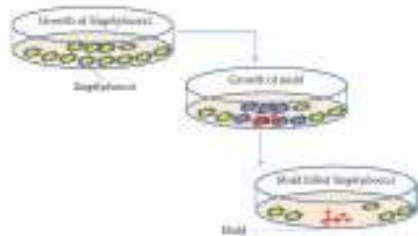
Surprisingly, with the help of genetic engineering, the gene of interest can be cut and inserted into the genome of other living organisms (microorganisms and viruses) and this process of gene insertion and manipulation is called recombinant DNA technology. Over the past few years recombinant DNA technology has been extensively employed to generate therapeutic products (insulin and growth hormones) for treating human diseases. With a rapid increase in the number of patients worldwide, there has been a tremendous scope to identify and create medicine for various human diseases. Thankfully with the availability of biotechnology tools, now it is possible to develop therapy for various diseases.

2-Medical products developed by using biotechnology tools:

Antibiotics

Penicillin is one of the earliest discovered antibiotics which is basically derived from molds such as *Penicillium*. Penicillin was discovered by Scottish scientist and Nobel laureate Sir Alexander Fleming in 1928. It all started with a basic experiment where Sir Fleming noticed a Petri dish containing *Staphylococcus* plate culture which he had mistakenly left open was contaminated by blue-green mold, which had formed a visible growth. But surprisingly he also found that there was a circle of inhibited bacterial growth around the mold. Later on, Sir Fleming hypothesized that the mold was releasing a substance that was preventing the growth and could contain a substance with antibiotic properties. In order to prove his hypothesis, he grew a pure culture and discovered the first antibiotic substance from the *Penicillium* mold, known as *Penicillium notatum*. Sir Alexander showed that *P. notatum* when grown in the appropriate substrate caused the release of chemical substances and these chemical

substances were named as antibiotics. He later on named that chemical substance as penicillin. Soon after this discovery, penicillin was considered to be the most effective drug against bacteria (Gram-positive bacteria), and not at all effective against Gram-negative bacteria. The discovery of penicillin marks the beginning of the antibiotic production and so far more than 200 different types of antibiotics have been produced.



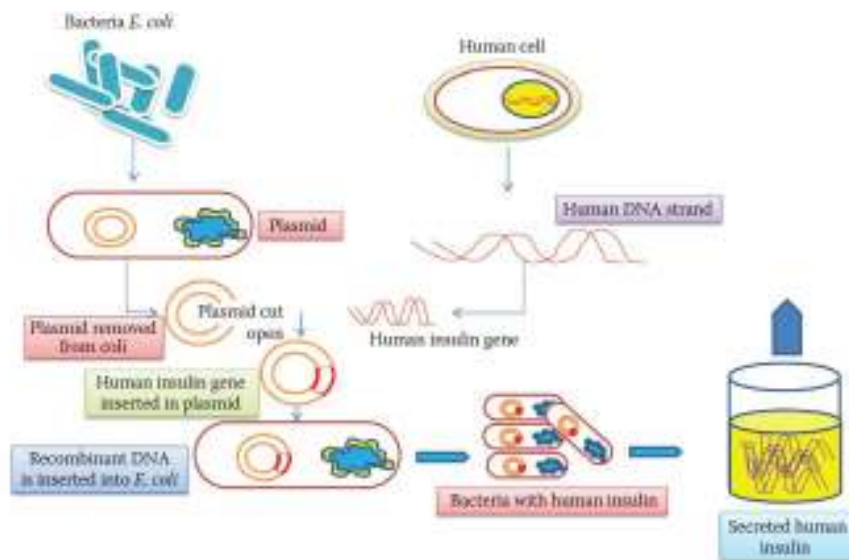
The pioneering experiment conducted by Dr. Alexander Fleming resulted in the discovery of the penicillin drug, an antibiotic drug.

3-Recombinant insulin

Another discovery which was made in the medical field was the recombinant DNA technology, which has completely revolutionized disease treatments especially in the Type 1 diabetes mellitus, where insulin-producing cells become dysfunctional and do not produce sufficient amount of insulin to regulate blood-sugar level. In a healthy human being, insulin regulates glucose metabolism in the body. In diabetic conditions, the level of insulin decreases which causes the elevation of the blood sugar level and this clinical condition is known as diabetes mellitus. One of the best treatments of diabetes mellitus is insulin injections, where insulin is injected into the patient's body and the insulin regulates the blood-sugar level. The insulin is either synthesized chemically or produced by recombinant DNA technology. These synthesized insulins are used medically to treat patients with Type 1 diabetes mellitus, whereas patients with Type 2 diabetes mellitus are insulin resistant.

Insulin Production -Advances

- Recently, human insulin gene into plants - safflower plant as bioreactors
- insulin analogues- chemically synthesized
- major hurdles - delivery of insulin - cannot be taken orally- improper absorption - biological activity lost.
- taken as subcutaneous injections - few companies oral form- trials are underway



Making of human insulin by recombinant DNA technology.

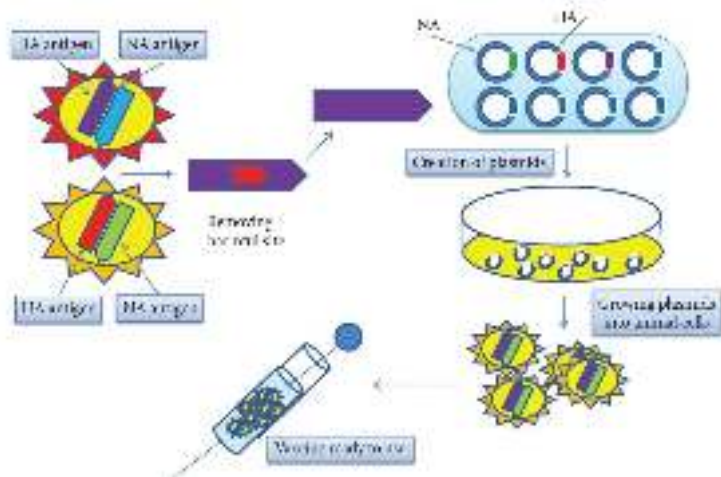
4-Vaccines

The vaccine is basically a biological preparation that improves the human immune defense system to fight diseases. The vaccines normally contain an agent that diligently resembles a disease-causing bacteria, and is frequently produced from debilitating or killed forms of the microorganisms. Upon injection into the human body, the vaccine can stimulate the body's immune system to recognize the agent as a foreign body, and recognize it, so that the immune system can recall and destroy or kill any of these microorganisms that later infect the human body. In a simpler way, the vaccine basically trains the human body to defend or fight or kill the microorganisms. The vaccines are classified based on its application; the vaccines that are used to prevent the effects of an upcoming infection by any natural or wild pathogen are called prophylactic, and the vaccines which are used against cancer are called therapeutic vaccines.

The story of vaccine development dates back to the seventeenth century when Edward Jenner found a milkmaid infected with smallpox, and a few days later he took pus from the hand of the milkmaid with cowpox and inoculated an eight-year-old boy with it. After six weeks he found that the boy did not contract smallpox. After a few years, Sir Louis Pasteur adapted Jenner's idea of developing a rabies vaccine and, subsequently, vaccine development progress started and became a matter of national concern. After that obligatory vaccination laws were passed in various countries around the globe. During the twentieth century, we have seen an introduction of several successful vaccines against diseases such as diphtheria, measles, mumps, and rubella. Interestingly, the major achievement was done in the 1950s when the polio vaccine was made and clinically used; also vaccines were synthesized to eradicate smallpox.

Successful vaccines

- Vaccines against diseases such as diphtheria, Anthrax, measles, mumps, and rubella, polio, smallpox, Hepatitis A, Hepatitis B, Flu etc



5-Monoclonal antibodies

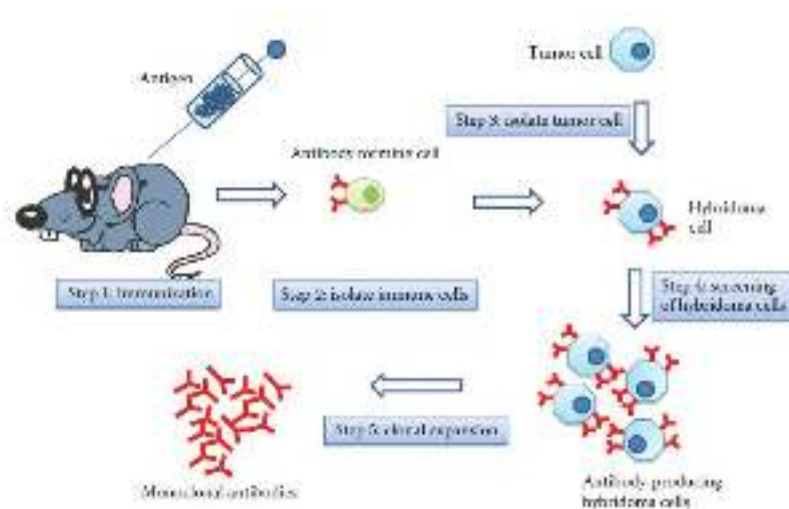
Another milestone achieved in the beginning of the twentieth century was the making of monoclonal antibodies, which was proposed by Paul Ehrlich. He suggested that drug compounds can be accurately, precisely, and specifically delivered along with monoclonal antibodies. Monoclonal antibodies are mono-specific antibodies as they are made by identical cells that are all clones of a distinctive parent cell. Currently, it is possible to produce monoclonal antibodies that specifically bind to that antigen and have a variety of applications, especially to detect or purify that substance (antigen), and has become an important tool in diagnostics and biomedical research.

In diagnostics and biomedical research, monoclonal antibodies are very useful tools. First, they are extremely specific; that is, each antibody binds to the specific site of the antigen. Second, some antibodies, once activated by the occurrence of a disease, continue to confer resistance against that disease; classic examples are the antibodies to the childhood diseases chickenpox and measles. Another important application of monoclonal antibodies is to develop vaccines against various diseases. As you are aware, a vaccine is made from bacteria or viruses either killed or inactivated. Upon introduction into the human body this vaccine stimulates the production of antibodies against the antigens to fight back the diseases. The production of monoclonal antibodies involves human and mouse hybrid cells and this technology is known as hybridoma technology. During monoclonal antibody production, tumor cells are merged with mammalian cells that produce an antibody against a particular antigen. The result of these merged tumors with mammalian cells is a called hybridoma, which can frequently produce antibodies. These antibodies are called monoclonal antibodies because they come from only one type of cell; whereas, the antibodies that are produced by conventional methods contain many kinds of cells and are called polyclonal antibodies.

Applications

- Diagnostic applications: e.g Quantification of blood hormones etc
- Therapeutic Applications: blocking the function of targeted molecule Protein Purification, Killing cancer cells

- Targeted Drug delivery
- Protein Purification



6-Bioengineered tissues

Over the last decade, tissue engineering became the most fascinating medical field, especially in body-parts reconstruction or cosmetic surgery areas. The basic concept about tissue engineering is to make human tissues through *in vitro* methods under controlled laboratory conditions and the making of such tissues is called bioengineered tissues. The definition of tissue engineering covers a broad range of applications in the healthcare field, however, in practice the term tissue engineering closely relates to repair or replacing portions of, or whole, human body tissues which include neurons, cardiomyocytes, bone, and cartilage. The bioengineered tissues are constructed by integrating certain mechanical and structural properties for proper functioning in the human body, so one can say that bioengineering is basically the use of a combination of cells, engineering, and biocompatible materials, and is finally suitable to improve or replace biological functions. The bioengineered field was once categorized as a sub-field of biomaterials, but having grown in scope and application it is considered as a field in its own right. Tissue engineering is an emerging multidisciplinary field involving biology, medicine, and engineering that is likely to revolutionize the ways we improve the health of millions of people worldwide by repairing, maintaining, or enhancing tissue and organ function. The tissue engineering can also have diagnostic applications where the human cells or tissues are used to test drug metabolism and drug uptake, toxicity, and pathogenicity.

In vitro cell culture techniques have successfully cultured and constructed the human cells outside of the body. These outside body constructed cells are often referred to as bioengineered cells. There are various applications of bioengineered cells in clinical conditions.

The term regenerative medicine is frequently used synonymously with cell and tissue engineering, though in regenerative medicine, cell-based therapy is considered to be a main branch of regenerative medicine.

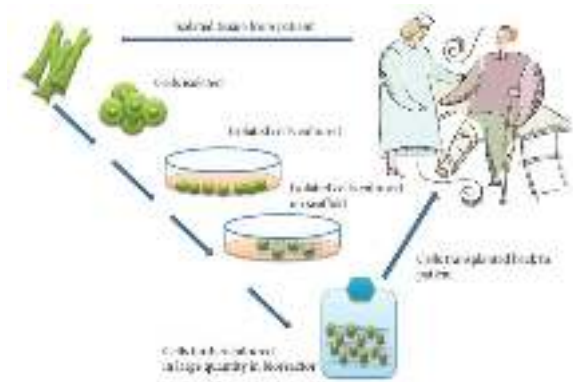
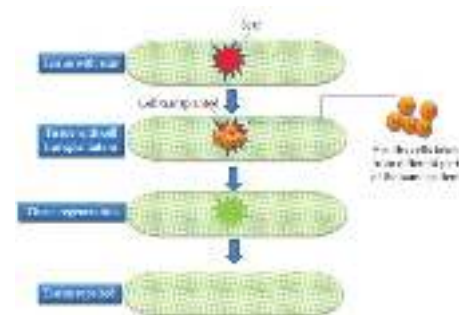
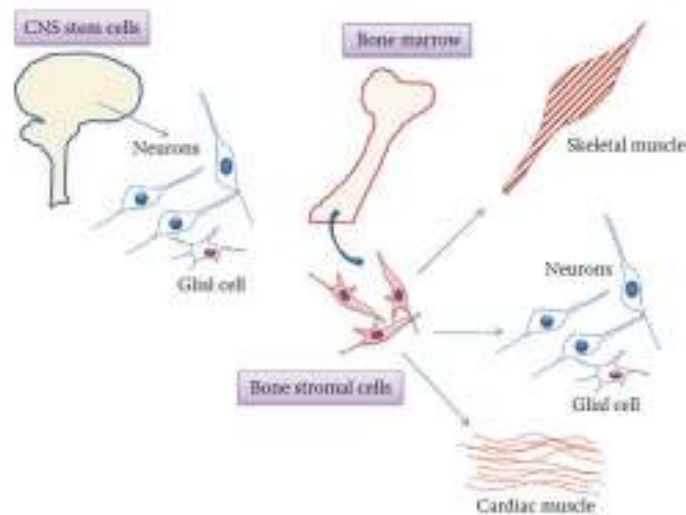


Figure. Cell and tissue engineering



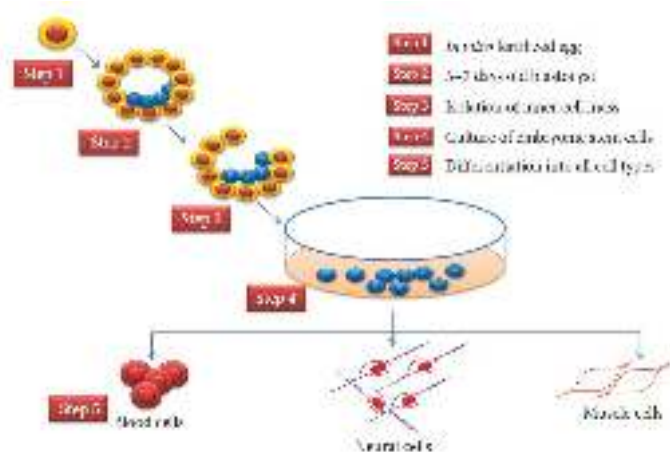
7-Adult stem cell therapy

Over the last few years, there has been tremendous interest in adult stem cells for the autologous cell transplantation as these specialized cells have the potential ability to repair or restore the dysfunctional cells or tissues in the human body afflicted with various diseases that include blood cancer and neurological disorders. The stem cells are generally found in all multicellular organisms and they are characterized by the ability to renew themselves through cell division and differentiate into a varied range of specialized cell types. Moreover, mammalian stem cells may be broadly classified into two major types: adult stem cells and embryonic stem cells (ESCs) that are isolated from the inner cell mass of blastocysts. One of the main roles of adult stem cells is that they remain in an undifferentiated state in the human body and multiply by cell division to replenish dying cells and restore damaged tissues and organs. The adult stem cells are also known as somatic stem cells; these stem cells can be found in both juvenile and adult ages. Moreover, adult stem cells are specialized cells that have the capability to divide and generate all cell types of the organ from which they originate, and can also possibly regenerate the entire organ. Unlike ESCs, the use of adult stem cells is not contentious as they are derived from adult source or tissue rather than by killing human embryos. Moreover, adult stem cells have been in use for many years predominantly in the treatment of cancer (such as leukemia and related bone and blood cancers) employing bone marrow transplants. Interestingly, the majority of the government funding especially in the United States is confined to adult stem cell-based research. Among adult stem cells, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) are found to be the most successful stem cells because they can be clinically used in patients. These stem cells can be directly injected or placed at the site of repair or injected through vascular delivery.



8-Emerging trends: Embryonic stem cells

Over the last few years ESCs have been a highly discussed and debated scientific topic at all levels which include public life, scientific forums, ethical, legal, and political platforms. Before we discuss the ethical or legal issues associated with ESCs, we will first learn what ESCs are all about. The ESCs are basically pluripotent stem cells isolated from the inner cell mass of the blastocyst, which is an early-stage embryo. Moreover, human embryos reach the blastocyst stage 4 to 5 days post fertilization, at which time they consist of about 50–150 cells. In addition to this, ESCs are adept at propagating themselves for an indefinite period under controlled culture situations. The culture and propagation of ESCs through in vitro methods is considered to be the best technology to culture human cells in large quantities and to be engaged as valuable tools for both research and cell-based therapy.



One of the best applications of ESCs is to provide a large supply of required cells to be used as cell-based therapy and tissue replacement in patients who are suffering from degenerative diseases. Moreover, a number of diseases that could possibly be treated by ESCs are cancers, diabetes, Parkinson's disease, Alzheimer's disease, stroke, blindness, and spinal cord injuries. In contrast to its various therapeutic applications, the issues related to ESCs need to be resolved before making them useful for human use. These issues are the problem of immune rejection associated with allogeneic stem cell transplantation. These problems can be solved

by using adult stem cells in autologous transplantation. Besides, cell-based therapy, the ESCs can be used as diagnostic tools to study an early human development and genetic disease, as well as in vitro drug testing. Other issues related to ESCs transplantation is that there is a possibility that transplanted stem cells could form tumors and have the possibility of becoming cancerous if cell division continues uncontrollably. Contrary to this, supporters of ESCs research argue that such research should be continued because the resultant research findings will have significant medical potential. The recent development of induced pluripotent stem cells (iPSc) has created tremendous interest among scientists, as adult stem cells can be converted into pluripotent stem cells and there is no need to kill the human embryos.

In spite of all controversies over ethical use of human ESCs, on January 23, 2009, Geron Corporation, USA, has received an approval from US Food and Drug Administration (FDA) to test ESCs in humans. The Phase 1 clinical trial for transplantation of a human-ES-derived cell population into spinal cord-injured individuals became the first human ES cell human trial. The Phase 1 clinical study was conducted on eight to ten paraplegic patients who have had their injuries no longer than two weeks before the trial began, and stem cells were injected before the formation of scar in the tissue. Nevertheless, the researchers are stressing that the injections are not expected to fully cure the patients and restore all mobility. The success of such clinical trials goes back to the experiments conducted by University of California, USA, and which was supported by Geron Corporation of Menlo Park (Biotechnology Company), California. The results of an animal study showed that there was functional improvement in locomotive recovery in spinal cord-injured rats after stem cell transplantation. Interestingly, the first trial is mainly for testing the safety of cell-based transplantation. However, the trial had been put on hold in August 2009 due to apprehensions made by the FDA regarding a small number of microscopic tumors found in several treated rat models, but the hold has been lifted since.

9-Human genome project

The Human Genome Project (HGP) was an international collaborative research project with a primary aim to decode the human DNA genome. The main objective of the HGP was to map about 20–25 k genes of the human genome from both a physical and functional perspective. The initial task of the project was to identify the full set of genetic instructions enclosed inside human DNA. The project began with the end of several years of work supported by the United States Department of Energy (DoE). It has been reported that the \$3-billion project was founded in 1990 by the DoE and the US National Institutes of Health. In addition to the United States, other countries also took part in the HGP, including the United Kingdom, France, Germany, Japan, China, and India. The team of scientists worked hard over the years and drafted a proposal for the genome project and the final sequencing of the human genome was done in 2006. Although the objective of the HGP is to understand the genetic organization of the human genome, the project has worked with other non-mammalian organisms.

Significance of the genome project

The reason for starting the HGP was to understand the role of genes in the development of diseases. There are reports that suggest more than 3000 genetic disorders known to be caused by genetic mutations. With the current treatment modalities, these genetic disorders cannot be cured without knowing the real cause. And to know the cause we have to know the role of genes in the diseases. Recent efforts have been made to find out the causes of cancer with genetic tools, but not much success was achieved. It has been recommended that information gained from the HGP would help to understand the genetic cause of the devastating illnesses, which include Parkinson's disease, schizophrenia, or Alzheimer's disease.

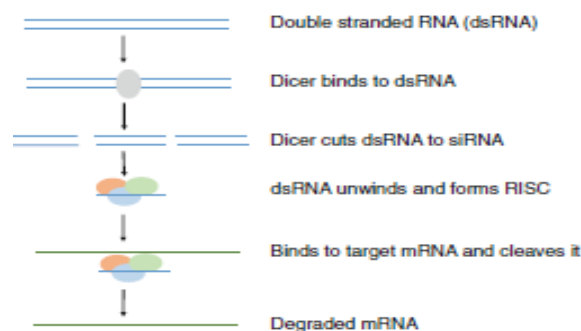
The genetic mutations play a role in many genetic diseases that include heart disease, diabetes, immune system disorders, and congenital defects. These diseases are thought to be the consequence from complex collaborations between genes and environmental factors. When genes for diseases have been identified, researchers can study how environmental factors, food, drugs, or pollutants can influence those genes. The location of the gene is important in identifying the type of protein which is produced by a particular gene. It has been reported that understanding the mechanism of a genetic disease is an important step in curing genetic diseases. It has been suggested that one day it may be possible to treat genetic diseases by gene therapy. Besides therapeutic applications, the information gained from the HGP can help to know the reason for the pluripotency nature of ESCs and how these ESCs can be differentiated into many different specialized cells such as muscle cell, neural cells, or hepatocytes.

10-RNA interference technology

The RNA interference (RNAi) is a natural process that cells dictate to turn down or go to a silent state; such activity cell is regulated by a specific gene known as RNAi. The RNAi technology provides excellent tools to study the role specific gene in the development, causes, and progression of particular diseases, and has taken the biomedical community by storm. It has been reported that RNAi was first noticed in the plant *Petunia* when plant biologists tried to develop the purple color of the flower by introducing a pigment-producing gene. Surprisingly, the gene which was introduced to intensify color in the plants was found to suppress the color. In the end the resulting flowers developed white patches or became completely white. Surprisingly, a few years later another group of researchers observed a similar kind of gene-silencing effect in *Caenorhabditis elegans*. Later on, it has been reported that the gene-silencing effect is caused by the presence of double-stranded RNA, and this double-stranded RNA is normally not found in healthy cells.

RNAi technology has been in use in various fields of biotechnology, particularly in the food plant engineering that produces lower levels of natural toxins. No plant that uses RNAi technology has yet passed the experimental stage; however, research work has been known to effectively reduce the allergen levels in tomato plants and also is known to cut the cancer-causing agents in tobacco plants. Also, other plant characters that have also been bioengineered include the production of nonnarcotic natural products by using the opium plant, development of resistance to common plant viruses, and protection of plants with dietary antioxidants. Interestingly, bioengineered plants such as *FlavrSavr* tomatoes and two cultivars of *ring-spot*-resistant papaya were originally developed by employing antisense

technology. One of the pronounced applications of RNAi technology is its application in medicine. Over the past few years, efforts have been made to understand RNAi's role in normal and diseased cells, and also to use the RNAi technology for use in medical therapies. It has been suggested that human disease progression can be blocked by using RNAi-based therapies to turn down the activity of genes. Cancer, for example, is frequently caused by over-excited genes in the cells, and retarding their activities could stop the disease progression. Over the last few years, several pharmaceutical companies are using RNAi-based therapies to treat for various forms of cancer. In addition, viral infections can also be treated using RNAi-based therapies and this can be done by reducing the activity of key viral genes. It has been shown in the laboratory that human cells have successfully stopped the growth of HIV, polio, hepatitis C, and other viruses in human cell culture and RNAi-based therapies against HIV are under clinical trial stages. Moreover, the importance of RNAi technology has some beneficial effects in finding out the cause of the disease. Using RNAi technology, the activity of a particular gene can be knocked down which will help us to understand its role in the disease development and progression. For many years, researchers have been studying how proteins regulate gene activity. Now with the help of RNAi technology, it would be possible to discover the role of proteins in gene regulation.

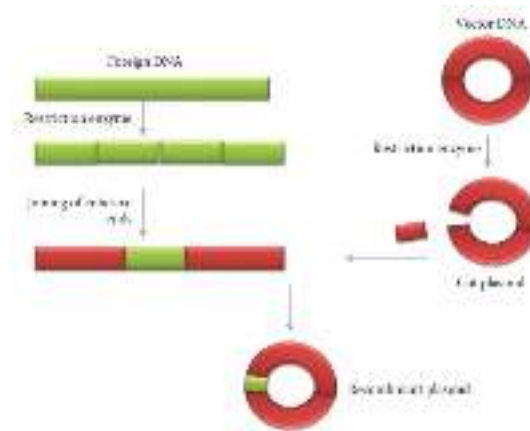


RNAi method for gene silencing. Dicer binds to dsRNA and cleaves it to small fragments. The fragments unwind and form RISC complex. The RISC complex binds to target mRNA and cleaves its sequence specifically.

11-Recombinant DNA technology

The process of r-DNA technology begins with the isolation of a gene of interest, which is then inserted into a vector; these vectors are further cloned into multiple copies. A vector is basically a piece of DNA that is capable of independent growth; bacterial plasmids and viral phages are the commonly used vectors. When the gene of interest (foreign DNA) is integrated into the plasmid or phage, this process is generally referred to as r-DNA. The next step in r-DNA technology is to introduce the vector containing the foreign DNA into host cells so that the cells can express the desirable proteins. In order to get sufficient amounts of protein, the vectors must be cloned to produce large quantities of the DNA. Once the vector is isolated in large quantities, it can be introduced into the desired host cells, which include mammalian, yeast, or special bacterial cells. Finally, the host cells can synthesize the foreign protein from the r-DNA. When the cells are grown in vast quantities in the bioreactor or

fermenter, the recombinant protein can be isolated in large amounts and the entire process is commonly referred as r-DNA technology. There are three different methods available through which r-DNA products are being developed: (1) transformation, (2) phage introduction, and (3) nonbacterial transformation.



Steps of constructing a recombinant DNA

The making of r-DNA has been briefly described below in a stepwise manner.

- Isolation of DNA
- Digestion of DNA
- Ligation of Two DNA Fragments
- Transferring Recombinant Vector into Host Cells (Transformation)
- Selection of the Transformed Cells

Goals of recombinant DNA technology

- To isolate and characterize a gene
- To make desired alterations in one or more isolated genes
- To return altered genes to living cells
- Artificially synthesize new gene
- Alternating the genome of an organism
- Understanding the hereditary diseases and their cure
- Improving human genome

Techniques used in rDNA technology

- Gel electrophoresis
- Cloning libraries
- Restriction enzyme mapping
- PCR
- Nucleic Acid Hybridization
- DNA Microarrays

Applications of rDNA technology

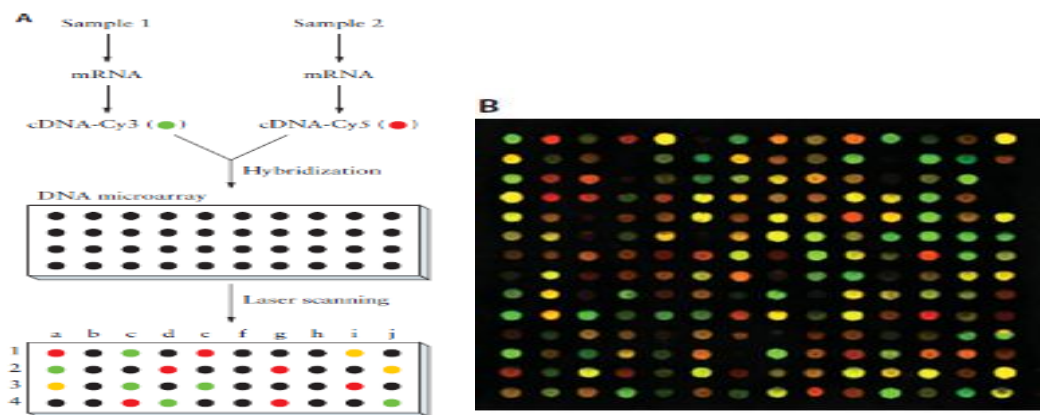
- Agriculture: growing crops of your choice (GM food), pesticide resistant crops, fruits with attractive colors, all being grown in artificial conditions

- Pharmacology: artificial insulin production, drug delivery to target sites
- Medicine: gene therapy, antiviral therapy, vaccination, synthesizing clotting factors
- Other uses: fluorescent fishes, glowing plants etc

12-Biochips

With the advent of information technology, it is possible to store biological information in a chip format which is called as a biochip. A microarray, also known as a DNA chip or biochip, is basically a collection of microscopic DNA spots attached to a solid surface (see picture). DNA microarray enables investigators to analyze expression of several genes in a single reaction. Typically it uses a solid surface such as a glass slide or a silicon film known as chip.

Gene expression profiling with a DNA microarray



(A) mRNA is extracted from two samples (sample 1 and sample 2), and during reverse transcription, the first cDNA strands are labeled with the fluorescent dyes Cy3 and Cy5, respectively. The cDNA samples are mixed and hybridized to an ordered array of either gene sequences or gene-specific oligonucleotides. After the hybridization reaction, each probe cell is scanned for both fluorescent dyes and the separate emissions are recorded. Probe cells that produce only a green or red emission represent genes that are transcribed only in sample 1 or 2, respectively; yellow emissions indicate genes that are active in both samples; and the absence of emissions (black) represents genes that are not transcribed in either sample. (B) Fluorescence image of a DNA microarray hybridized with Cy3- and Cy5-labeled cDNA

Applications

DNA microarray has increasing number of applications:

1-Expression analysis : In this method, the DNA of an organism is spotted on a chip and hybridized to cDNA obtained from mRNA of the same organism. The intensity signifies the abundance of a particular mRNA in the sample, signifying the expression level of the gene under a given condition. This is the most commonly used technique.

2-Mutation analysis : The genomic DNA is spotted on a chip and hybridized to DNA isolated from different individuals. Single nucleotide polymorphism can be identified by this method. It is important to analyze variation in a particular gene.

3- Comparative genomic hybridization: Increase or decrease of chromosomal fragments associated with a disease state. Prenatal chromosomal aberrations can be studied.

13-Gene therapy

To treat genetic disorders, the default or dysfunctional gene can either be replaced with a new gene or repaired with gene therapy. In brief, the gene therapy involves correcting defects in genes and in this process a normal/healthy gene is introduced to replace a defective gene. A person carrying the defect in a specific gene might develop disease, which may be inheritable. In gene therapy, nucleic acid is the therapeutic agent.

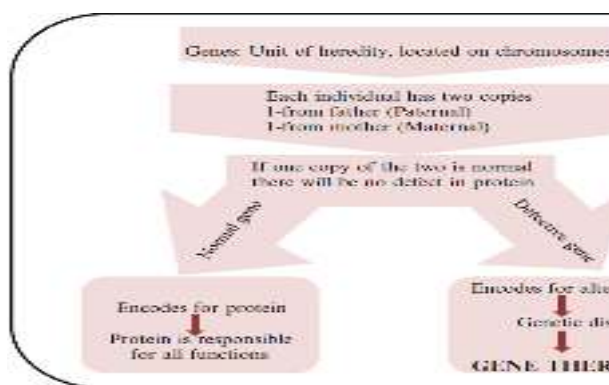
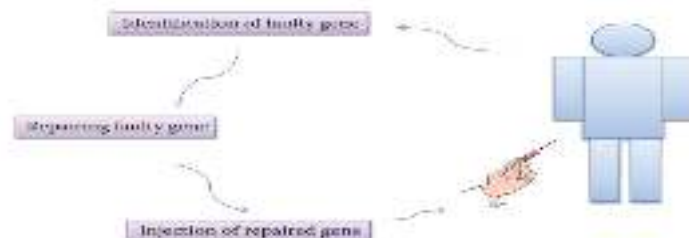


Fig. The function of normal gene and effect of defective gene, which causes disease. The individuals with defective gene are the candidate for gene therapy.

Gene therapy is still under development stages and various clinical trials are underway around the world. Its success will be based on the successful cure of genetic diseases by gene therapy.

The therapy is achieved by using a viral vector or gene delivery vehicles. The steps involved in the therapy in which the gene of interest enters the target cell via viral vector and begins expression are referred to as transduction.



The gene therapy can be classified into two major types: somatic gene therapy and germline gene therapy.

Somatic gene therapy

By using somatic gene therapy, somatic cells can be treated by inserting a vector loaded with the correct gene into a person's body. The somatic cells are cells that form in the body and cannot produce progenies. Gene therapy, in its present stage only treats somatic cells in humans.

There are two types of somatic gene therapy, *ex vivo* and *in vivo*.

- **Ex vivo somatic gene therapy:** In *ex vivo* gene therapy genes or cells are modified outside the body and then transplanted back into the body.
- **In vivo gene therapy,** cells are modified or treated within the patient's body.

Germline Therapy

In the germline therapy, the modification is done in germ cells that are sperms or eggs, producing a permanent and genetically transferable modification. The functional gene is introduced into either of them, which then integrate in the selected germ cell, and subsequently the therapeutic gene is present in each cell of the body. The fertilized egg may be used to insert the therapeutic gene and reimplanted into the mother. If successful, the functional gene is present and expressed in all cells of the resulting individual. The technique of making permanent changes might prove effective in the genetic diseases, but due to ethical and technical reasons, their use has been prohibited in humans.

Importance of vectors in gene therapy

Vectors should be undetected by the body's immune system to avoid immune rejection. Most viruses attack their hosts to insert their genetic material into the DNA of the host and this DNA contains instructions to produce viruses in large numbers. Considering these capabilities, scientist thought of using these viruses as a vehicle to deliver genetic materials to the host cells in order to treat genetic diseases. Currently the most common vector is used as a vehicle virus that has been genetically altered to carry normal human DNA. We have described various types of viral vectors which are being used in gene therapy and these viruses are found to be different in their mechanisms of action.

To make gene therapy effective, there are two classes of viruses—retroviruses and adenoviruses—found to be critical in gene delivery.

Recommendation to resolve the Legal and ethical issues

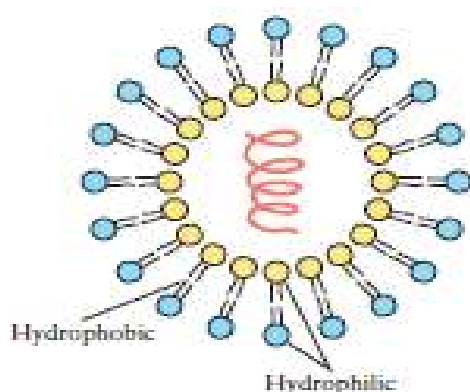
Nevertheless, there are some ethical concerns which have been raised in different parts of the world, and some ethical organizations have released recommendations to resolve the gene therapy issues and these recommendations include (a) establishing a national ethics body in each country to look at somatic gene therapy; (b) supporting somatic gene therapy research that follows the recommendations; (c) asking researchers, organizations, and governments to listen and respond to public concerns about gene therapy research; and (d) asking that research follows quality and safety controls.

14-Liposome-Based Drug Delivery

Liposomes are artificially prepared vesicles made of lipid bilayer. Liposomes can be filled with drugs, and used to deliver drugs for cancer and other diseases. Liposomes have liquid core surrounded by phospholipid bilayer. Liposomes can be formed by sphingomyelins or lecithins and synthetic lipids as dimyristoyl, distearoyl, dipalmitoyl, and dioleoyl. They are 10 nm to several-micrometer spherical nanostructured molecules with defined shape and size. Liposomes are formed by an external phase having double phospholipid membranes and an aqueous internal phase with the capability to encapsulate both hydrophobic and hydrophilic compounds. Their advantages are biocompatibility, low toxicity, non- immunogenicity, and improved drug efficacy. They fuse to cell membrane and deliver the drug.

They are used as carriers of antifungal, antibacterial, and cancer drugs:

- Liposome is used to deliver anticancer drug doxorubicin. Doxorubicin can induce myocardial toxicity. However when delivered in a liposomal preparation, the off-target toxicity is significantly reduced.
- Liposomes consisting of phosphatidylcholine, phosphatidylethanolamine, oleic acid, and cholesteryl hemisuccinate were developed by encapsulating a purified recombinant T4 endonuclease V . When delivered to the skin, they tend to improve DNA repair.



Structure of liposomes. They have liquid core surrounded by phospholipid layer. They are biocompatible, nontoxic, and non-immunogenic and used for packaging of drugs such as amphotericin B, ampicillin, polymyxin B, ciprofloxacin, anticancerous (daunorubicin and doxorubicin), insulin, and DNA. Used for drug delivery in fungal and bacterial infections, cancer cells, and diabetes.

15-Bionanotechnology

Bionanotechnology is a term that refers to the intersection of nanotechnology and biology.

This discipline helps to indicate the merger of biological research in various fields of nanotechnology.

Nanomaterial is the material used to make or construct biomaterials with nanomorphological features and these biomaterials can be used for various applications. These nanomaterials are basically smaller than 1/10th of a micrometer in at least one dimension; however, this term is occasionally used for materials smaller than 1 μm .

Application of nanomaterials in medicine

Therapeutic delivery: It has been reported that drug needs to be transported well in the body to easily reach the target site and there are times some drugs are not able to reach to target sites and get lost in the process. Recent efforts have been made to improve drug bioavailability, and nanomaterials play an important role in it. The word “bioavailability” denotes the presence of drug molecules where they are needed in the body and perform normal body function.

Nanoparticles can also be used as contrastagents, to take ultrasound and magnetic resonance imaging (MRI) pictures. Additionally, polymer-based nanoparticles can be designed to improve the pharmacological and therapeutical properties of drugs.



Figure- Applications of nanotechnology in medicine

The concepts that are enhanced through nanobiology include: nanodevices, nanoparticles, and nanoscale that occurs within the discipline of nanotechnology. Moreover, this technical method in biology allows scientists to imagine and create systems that can be used for biological research. Biologically inspired nanotechnology uses biological systems as the inspiration for technologies not yet created. The most important objectives that are frequently found in nanobiology involve applying nanotools to relevant medical or biological problems and refining these applications. Moreover, developing new tools for the medical and biological fields is another primary objective in nanotechnology.

Despite the fact that biological systems are inherently nano in scale, nanoscience must merge with biology in order to deliver biomacromolecules and molecular machines that are similar to the human body or organ. In the twenty-first century, scientists have developed the technology to artificially tap into nanobiology. This process is best described as organic merging with synthetic. Interestingly, colonies of live neurons can live together on a biochip device. The self-assembling nanotubes have the ability to be used as a structural system as they would be composed together with rhodopsins, which would help the optical computing process and also help with the storage of biological materials. The most fascinating aspects would be of DNA as the software for all living things, which can be used as a structural proteomics system—a logical component of molecular computing.

16-Chromosomal Abnormalities

All nucleated cells contain chromosomes that consist of DNA and proteins (histones) in a compact structure. Chromosomes carry all of our genes and therefore all of our genetic information. DNA with its associated packaging proteins is referred to as **chromatin**. Some regions of chromosomes are tightly packed and are called heterochromatin, while other regions are less condensed and are called euchromatin. Less condensed packing of chromatin generally increases the transcription of genes in the region. Each species has a characteristic number and form of chromosomes, referred to as the **karyotype**. A **karyogram** is a photographic representation of stained chromosomes arranged in order of size, i.e., decreasing length (Fig. 1).

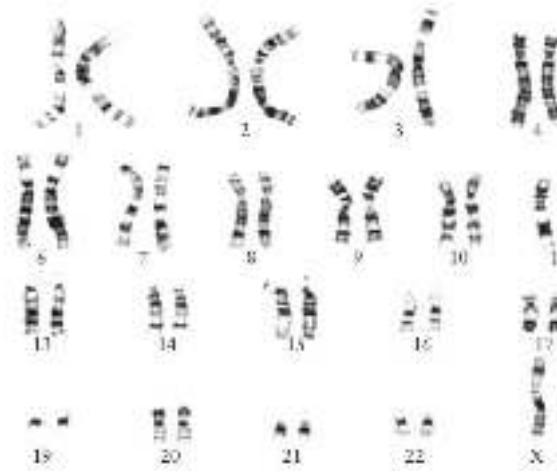


Fig. 1 Normal human male 46,XY karyogram. Humans have a total of 46 chromosomes that consist of two identical sets of 22 chromosomes (autosomal chromosomes) and 2 sex chromosomes (XX for female and XY for male). Because of the two identical sets of chromosomes, human cells are called diploid.

Each chromosome is paired with its matched or **homologous chromosome**. The matched chromosomes are identical in size and structure but may carry different versions (known as alleles) of the same gene. Humans have 46 chromosomes, or 23 chromosome pairs, to carry our approximately 25,000 genes. Cells in our body that contain 46 chromosomes are **diploid** ($n = 2$); 23 chromosomes are derived from the mother's egg cell, and the other 23 are from the father's sperm. Egg cells and spermatozoa each contain only 23 chromosomes (Fig. 2) and therefore are **haploid** ($n = 1$). In diploid cells, the 46 chromosomes appear as 22 homologous pairs of **autosomes** (nonsex chromosomes) and one pair of sex chromosomes, XX in females and XY in males.

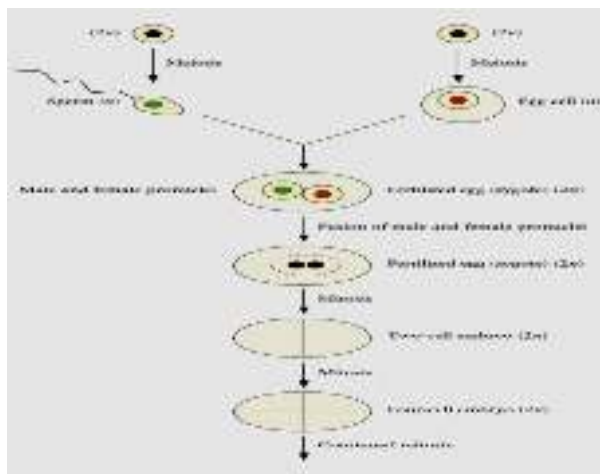


Fig.2 Generation of a diploid ($2n$) zygote. A diploid $2n$ fertilized egg (zygote) is produced by the fusion of a haploid (n) sperm and egg. Successive mitotic divisions generate many types of diploid cells in the body (somatic cells) during development and cell turnover (replacement of old cells with new ones) in an embryo.

Human chromosomes are usually studied in rapidly dividing cells, such as peripheral blood lymphocytes. Cell mitosis can be arrested in the metaphase stage of the cell cycle, and the chromosomes can be differentially stained to allow their identification microscopically. This **microscopic analysis of chromosomes is known as cytogenetics**. For routine karyotyping, Giemsa staining is preferred, as this procedure produces alternating light and dark bands (**G banding**) that reflect differential chromosomal structures characteristic of each chromosomal pair (Fig. 1). These light and dark bands of chromosomes result from the specificity of binding of the Giemsa stain for the phosphate groups of DNA, as the stain attaches to regions of DNA where there are large amounts of A-T bonding. Thus, Giemsa staining can identify different types of changes in chromosomal structure as gene rearrangements.

Examination of a karyotype enables one to determine either if there is gain or loss of a chromosome(s) or if the structure of a given chromosome(s) is altered. The **centromere** of each chromosome separates the short arm (p) from the long arm (q). Most arms are divided into two or more regions by distinct bands, and each region is further subdivided into subbands (Fig. 3). For example, band Xp21.2 is found on the p arm of the X chromosome in region 2, band 1, subband 2. **Chromosome disorders** are caused by abnormalities in the number (increase or decrease of genes) or the structure of chromosomes. An individual's physical characteristics are called a **phenotype**, which is the combination of all of that individual's expressed traits, including morphology, development, behavior, and biochemical and physiological properties. Phenotypes result from the expression of genes and environmental factors that interact with these genes. Thus, the phenotype of a person with a chromosomal disorder may vary with the type of chromosomal defect.

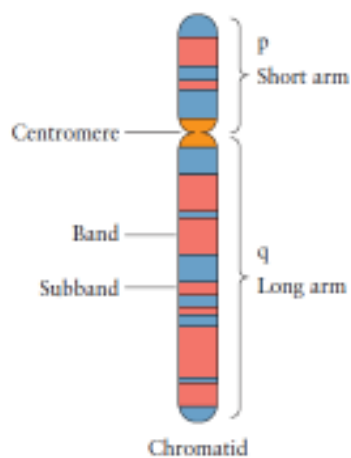


Fig.3 Schematic diagram of the structures of a chromosome. The p and q arms, centromere, chromatid, bands, and subbands (described in the text) are shown.

A chromatid is an individual chromosome that is paired with a replicated copy of the identical chromosome. This pair of chromosomes is held together at the centromere for the process of cell division. The light and dark bands originate from the differential staining of the regions of the chromosome with the Giemsa stain, as explained in the text. The short arm of each chromosome is denoted with a “p” and the long arm with a “q.” Thus, 7q refers to the long arm of chromosome 7. Each arm may be further divided into regions, depending on the size.

Risk factors for chromosomal abnormalities:

- Radiation
- Smoking
- Fertility drugs
- Alcohol consumption
- Oral contraceptive

17-Chromosome Structural Abnormalities

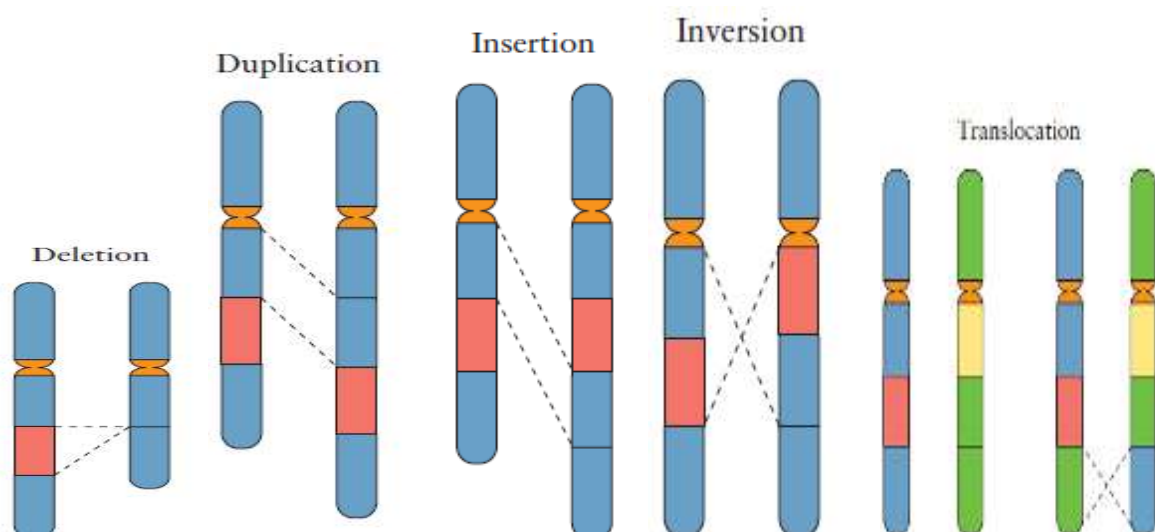
Genomic rearrangements may alter genome architecture and yield clinical consequences. Several genomic disorders caused by structural variation of chromosomes were discovered by cytogenetics. Recent advances in molecular cytogenetic techniques have enabled the rapid

and precise detection of structural rearrangements on a whole-genome scale. This high resolution illustrates the role of **structural variants (SVs)** and **single-nucleotide polymorphisms (SNPs)** in normal genetic variation.

In analyzing the role of structural gene variants in cell function, it is important to consider the two types of such variants, i.e., **gain-of-function variants** and **loss-of-function variants**. A gain-of-function variant results from a mutation that confers new or enhanced activity on a protein. Most mutations of this type are not heritable (germ line) but, rather, are somatic mutations. A change in the structure of a gene that may arise during DNA replication and is **not inherited from a parent, and also is not passed to offspring, is called a somatic mutation**. Such a mutation that results in a single base substitution in DNA is known as a somatic point mutation.

A loss-of-function variant results from a point mutation that leads to reduced or abolished protein function. Most loss-of-function mutations are recessive, indicating that clinical signs are typically observed only when both chromosomal copies of a gene (one being inherited from each parent) carry such a mutation.

In a population, any two unrelated individuals are identical at about 99.5% of their DNA sequence. At a given chromosomal site, one individual may have the A nucleotide and the other individual may have the G nucleotide. This type of site in DNA is known as an **SNP**. Each of the two different DNA sequences at this site (or gene) is called an **allele**. Molecular techniques such as array-based **comparative genomic hybridization (CGH)**, SNP arrays, array painting, and **next-generation sequencing** have facilitated and expedited the characterization of chromosome rearrangements in human genomes. These various genomic rearrangements can arise by several mechanisms, including deletions, amplifications, translocations, and inversions of DNA fragment. **Deletions** occur when a portion of a chromosome is missing or removed. **Duplications** result from the copying of a portion of a chromosome that results in extra genetic material. During a **translocation**, a portion of one chromosome is transferred to another chromosome. In a reciprocal translocation, DNA segments from two different chromosomes are exchanged. A DNA **inversion** results when a portion of a chromosome is broken off, turned upside down, and reattached. When a portion of a chromosome is disrupted, the chromosome may form a circle, or ring, without any loss of DNA.



18-Chromosome Numerical Abnormalities

An abnormality in which the chromosome number is an exact multiple of the haploid number ($n = 23$) and is larger than the diploid number ($n = 46$) is called **polyploidy**. Polyploidy arises from fertilization of an egg by two sperm (total number of chromosomes increases to 69) or the failure in one of the divisions of either the egg or the sperm so that a diploid gamete is produced. The survival of a fetus to full term of pregnancy is rare in the instance of polyploidy.

Aneuploidy occurs when the chromosome number is not an exact multiple of the haploid number and results from the failure of paired chromosomes (at first meiosis) or sister chromatids (at second meiosis) to separate at anaphase. Thus, two cells are produced, one with a missing copy of a chromosome and the other with an extra copy of that chromosome (Fig. 1). Examples of numerical chromosomal abnormalities are listed in Table 1.

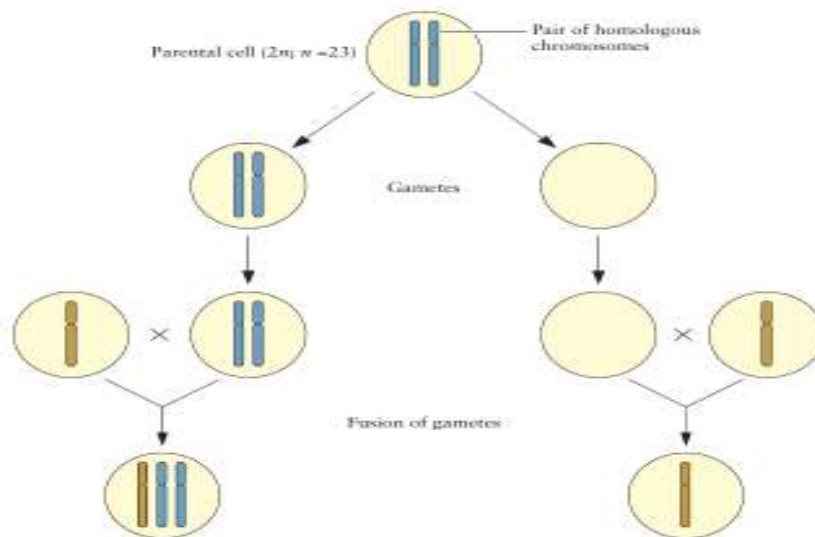


Fig.1 Nondisjunction in gamete cell formation and fusion of abnormal gametes with a normal haploid gamete. Aneuploidy occurs when paired chromosomes (at first meiosis) or sister chromatids (at second meiosis) do not separate from each other at anaphase, a stage of meiosis at which sister chromosomes move to opposite sides of the cell. This failure of paired chromosomes to separate, with the chromosomes instead moving to the same side of the cell, is termed nondisjunction. As a result, two cells are produced, one with a missing copy of a chromosome and one with an extra copy of that chromosome, as shown.

Table-1 Numerical chromosomal aberration syndromes

Aneuploidy condition	No. of chromosomes	Karyotype
Tetraploidy	92	XXYY
Triploidy	69	XXY
Trisomy 21 (Down syndrome)	47	XX+21
Trisomy 18 (Edward syndrome)	47	XY+18
Trisomy 13 (Patau syndrome)	47	XX+13
Klinefelter syndrome	47	XXY
Trisomy X	47	XXX
Turner syndrome	45	X

Trisomy 21, the first human chromosomal disorder discovered (in 1959), is an abnormality that displays an extra copy (total of 3 copies) of chromosome 21 (Fig. 2) and causes **Down syndrome**. The genes on all three copies of chromosome 21 are normal. However, not all individuals with Down syndrome show the same physical characteristics, indicating that their phenotypes can vary. People with Down syndrome have a typical facial appearance (the face is flat and broad) that includes an abnormally small chin, skin folds on the inner corners of the eyes, poor muscle tone, a flat nasal bridge, a protruding tongue due to a small oral cavity, an enlarged tongue near the tonsils, a short neck, and white spots on the iris. Growth parameters such as height, weight, and head circumference are smaller in children with Down syndrome than in typical individuals of the same age.

All Down syndrome patients have some degree of mental retardation, albeit moderate. Despite this condition, many persons with Down syndrome can be educated and live with minimal daily assistance, while others require much attention and care. There are several possible health concerns, including cardiac failure and hearing loss. Individuals with Down syndrome are now living longer than they used to and can survive into their 50s and 60s. However, those individuals who survive to that age are at very high risk of developing Alzheimer disease.

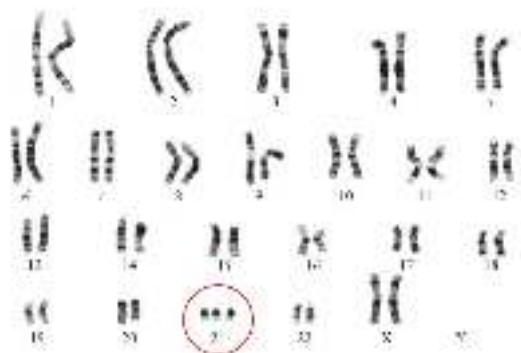


Fig-2 Trisomy 21. This syndrome appears when an individual inherits three copies of chromosome 21. The extra copy of chromosome 21 results in Down syndrome.

Trisomy 13, the presence of three copies of chromosome 13, causes **Patau syndrome**. Only about 5% of infants with this disorder survive past their first year, and most pregnancies involving trisomy 13 end in miscarriage. Children with trisomy 13 usually have a lot of trouble breathing, especially when they sleep, and many have seizures. All individuals with Patau syndrome have severe mental retardation, and other common characteristics include a small head, extra fingers and/or toes, and a cleft lip or cleft palate.

Trisomy 18, the presence of 3 copies of chromosome 18, elicits **Edward syndrome**. Only about 10% of babies with this syndrome survive past their first year, and the majority of survivors are female, indicating a prenatal selection against males with trisomy 18 after the time of amniocentesis. Children with trisomy 18 usually have problems with breathing and eating, and many have seizures or serious heart conditions. All individuals with trisomy 18 have severe mental retardation. Most babies with trisomy 18 are very small and have certain recognizable facial features. They also tend to overlap their fingers in a very distinct pattern.

Prader-Willi syndrome results from the absence or nonexpression of a group of genes on chromosome 15. A specific form of blood cancer, **chronic myeloid leukemia**, may be caused by a chromosomal translocation, in which portions of two chromosomes (chromosomes 9 and 22) are exchanged. No chromosomal material is gained or lost, but a new, abnormal gene that leads to the development of cancer is formed.

19-Sex Chromosome Abnormalities

The phenotype of chromosomal disorders can vary depending on whether the chromosomal abnormality occurs on the maternally or paternally derived chromosomes. **Turner syndrome** (45,X) is an example of monosomy, in which a girl is born with only one sex chromosome, an X chromosome.

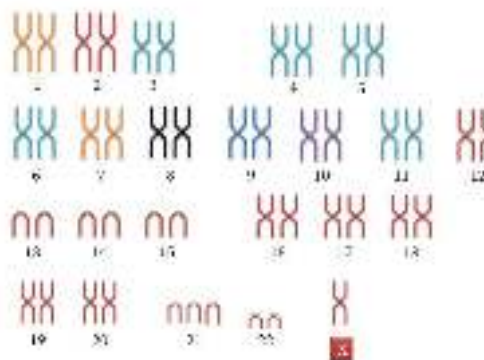


Fig- Turner syndrome (45 X).

Klinefelter syndrome occurs in males with the genotype 47,XXY. Such males have 47 chromosomes and are classified as having a sex chromosome trisomy (three sex chromosomes), since they carry two X chromosomes and one Y chromosome. This syndrome affects about 1 in 1,000 males. Most affected males are taller than average, and they may have more body fat in the hips or chest as well as little facial and body hair. Some Klinefelter males are mentally retarded, while many others have normal intelligence. The most common feature of this syndrome is infertility; about 2% of infertile men have Klinefelter syndrome.

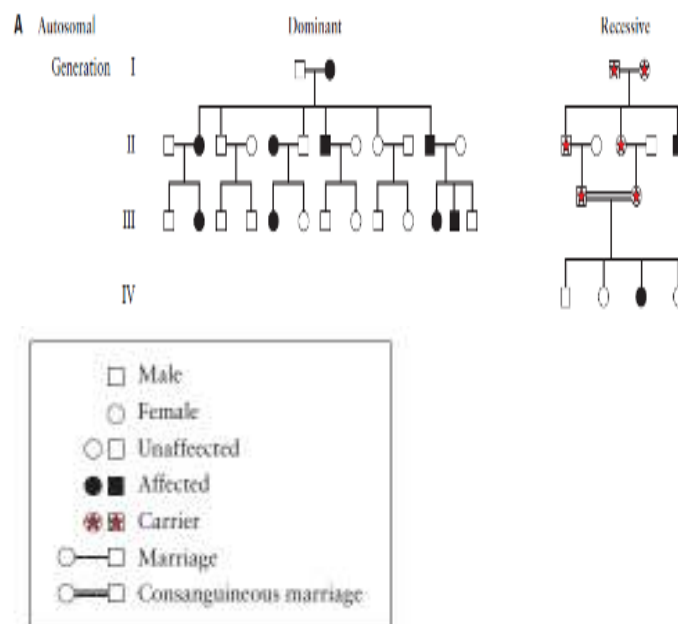
20-Single Gene Disorders

The genetic disorder in which a single gene gets mutated is called as a single-gene disorder. When a gene is mutated so that its protein product cannot carry out its normal body function, it leads to the development of a disorder.

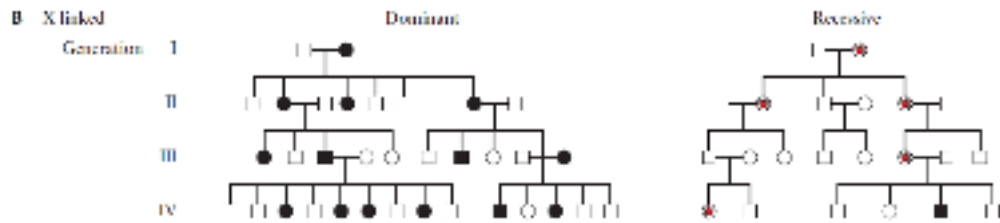
Single-gene disorders, or monogenic diseases, result from a mutation(s) in a single gene occurring in all cells of the body (somatic cells). Inheritance of these types of disorders

follows a Mendelian segregation pattern. Although very rare, such disorders affect millions of people globally. Currently, it is estimated that more than 10,000 human diseases are monogenic. These diseases can occur in about 1 out of every 100 births and thus can cause a significant loss of life. So-called “pure genetic diseases” are caused by a single nucleotide change in a single gene in human DNA. The nature of disease depends on the functions performed by the modified gene. Monogenic diseases can be classified into three main categories: **autosomal dominant**, **autosomal recessive**, and **X linked**.

All humans have two chromosomal copies of each gene or allele, one allele per each member of a chromosome pair. Dominant monogenic disorders involve a mutation in only one allele of a disease-related gene. In autosomal dominant inheritance, an affected person has at least one affected parent, and affected individuals have a 50% chance of passing the disorder on to their children. Huntington disease is an example of an autosomal dominant disorder.



Recessive monogenic disorders occur due to a mutation in both alleles of a disease-related gene. In autosomal recessive inheritance, affected children are usually born to unaffected parents. Parents of affected children usually do not have disease symptoms but carry a single copy of the mutated gene. There is an increased incidence of autosomal recessive disorders in families in which parents are related. Children of parents who are both heterozygous for the mutated gene have a 25% chance of inheriting the disorder, and the disorder affects either sex. Cystic fibrosis and sickle-cell anemia are examples of autosomal recessive disorders. X-linked monogenic disorders are linked to mutations in genes on the X chromosome. The X-linked alleles can also be dominant or recessive. These alleles are expressed in both men and women, more so in men, as they carry only one copy of the X chromosome (XY), whereas women carry two (XX).



Y-linked inheritance would affect only males, the affected males would always have an affected father, and all sons of an affected man would have the disease. However, no Y-linked diseases have ever been discovered. Apart from male infertility, mutations in Y-linked genes may not give rise to any disorders.



The prevalence of some monogenic inheritable disorders is shown in Table

Disorder	Prevalence (approximate)
Autosomal dominant	
Familial hypercholesterolemia	1 in 500
Polycystic kidney disease	1 in 1,250
Neurofibromatosis type I	1 in 2,500
Hereditary spherocytosis	1 in 5,000
Marfan syndrome	1 in 4,000
Huntington disease	1 in 15,000
Autosomal recessive	
Sickle-cell anemia	1 in 625
Cystic fibrosis	1 in 2,000
Lysosomal acid lipase deficiency	1 in 40,000
Tay-Sachs disease	1 in 3,000
Phenylketonuria	1 in 12,000
Mucopolysaccharidoses	1 in 25,000
Glycogen storage diseases	1 in 50,000
Galactosemia	1 in 57,000
X linked	
Duchenne muscular dystrophy	1 in 7,000
Hemophilia	1 in 10,000

21-CYSTIC FIBROSIS

Disorder and Genetics:

Cystic fibrosis is a genetic disorder that affects the respiratory, digestive, and reproductive systems. This disorder is mediated by the production of abnormally thick mucous linings in the lungs, can lead to fatal lung infections, and can give rise to obstruction of the pancreas and impair digestion. The severity of the condition varies from the mild forms (e.g., absence of the vas deferens in men) to the more common severe forms (epithelial gland dysfunction). Since an individual must inherit two defective cystic fibrosis genes, one from each parent, to get the disease, cystic fibrosis is classified as an autosomal recessive disorder.

In families in which both parents are carriers of the cystic fibrosis gene, there is a 25% chance that they will transmit cystic fibrosis to their child, a 50% chance that the child will carry the cystic fibrosis gene, and a 25% chance that the child will be a non carrier.

The cystic fibrosis gene encodes a protein known as the cystic fibrosis transmembrane regulator (CFTR), and the *CFTR* gene contains 27 exons within 250 kb of genomic DNA. The CFTR protein consists of 1,480 amino acids distributed in two membrane-spanning domains and two ATP-binding domains.

The membrane-spanning domains form a low-conductance cyclic adenosine monophosphate (cAMP)-dependent chloride channel. The ATP-binding domains control channel activity.

The most common mutation, a 3-bp deletion at codon 508 ($\Delta F508$), results in the loss of a phenylalanine residue from the first ATP-binding domain and blocks the transport of the CFTR protein to the epithelial cell membrane.

The next most common mutation ($\leq 5\%$ of *CFTR* mutations) is also present in the first ATP-binding domain and G551D (glycine at residue 551 is replaced by aspartic acid) in exon 11. Both common mutations are associated with a severe form of the disease in the homozygous state.

Prevalence

Cystic fibrosis is a common cause of death in childhood and the most common inherited disease in white populations. The incidence of cystic fibrosis varies significantly in different countries: 1 in 2,500 births in the United Kingdom, 1 in 2,000 to 3,000 births in Europe, and 1 in 3,500 births in the United States. Cystic fibrosis can arise in nonwhite populations, but only very rarely (1 in 100,000 births in African-American and East Asian populations).

Diagnosis and Prognosis

Cystic fibrosis patients have many symptoms (salty skin; persistent coughing, wheezing, or shortness of breath; and excessive appetite but poor weight gain), and their symptoms may vary due to the >200 mutations of the *CFTR* gene.

A diagnostic sweat test measures the amount of salt in sweat of patients with cystic fibrosis (a high salt level indicates cystic fibrosis). This test is usually performed in babies older than 3 to 4 weeks and can also confirm the diagnosis in older children and adults.

If pancreatic enzyme levels are reduced, stool analyses may reveal decreased or absent levels of the digestive enzymes (trypsin and chymotrypsin) or high levels of fat. If insulin secretion is reduced, blood sugar levels are high. Lung function tests may show that breathing is compromised.

Genetic testing on a small blood sample can help determine whether an individual has a defective *CFTR* gene. During pregnancy, an accurate diagnosis of cystic fibrosis in the fetus is possible.

22-Fragile X Syndrome

Disorder and Genetics: Fragile X syndrome is caused by a “fragile” site at the end of the long arm of the X chromosome. This syndrome is manifested by many changes in behavior and cognitive recognition that vary widely in severity among patients.

Fragile X syndrome is the most common cause of inherited mental retardation. Although it is an X-chromosome-linked recessive trait with variable expression and incomplete penetrance, 30% of all carrier women are affected.

Penetrance is the proportion of individuals carrying a particular variant of a gene (allele or genotype) who also express an associated trait or phenotype. Full penetrance occurs when all individuals carrying a gene express the phenotype. Incomplete penetrance occurs when some individuals fail to express the phenotype, even though they carry the variant allele.

Fragile X syndrome is caused by loss-of-function mutations in the fragile X mental retardation 1 (*FMR1*) gene. *FMR1* encodes the FMRP protein found in many tissues and at particularly high levels in the brain and testes. In the brain, it may play a role in the development of neuronal synapses and cell communication. The synapses can change and adapt over time in response to experience, a characteristic called **synaptic plasticity**. The FMRP protein may help regulate synaptic plasticity and thereby control learning and memory.

Fragile X syndrome belongs to a growing class of **neurodegenerative disorders** known as **trinucleotide repeat disorders**. Among these disorders, 14 affect humans and elicit neurological dysfunction. Trinucleotide CGG repeat expansions (200 to more than 1,000 repeats) that inactivate the *FMR1* gene are the most common mutations observed at this locus.

Prevalence

Fragile X syndrome is the single most common inherited cause of mental impairment, affecting 1 in 3,600 males and 1 in 4,000 to 6,000 females worldwide. Approximately 1 in 259 women of all races carry the fragile X gene and may pass it to their children, whereas about 1 in 800 men of all races and ethnicities are carriers. Carrier females have a 30 to 40% chance of giving birth to a mentally retarded male child and a 15 to 20% chance of having a mentally retarded female child.

Diagnosis and Prognosis

The diagnosis of fragile X syndrome is made by the detection of mutations in the *FMR1* gene. Over 99% of individuals have an *FMR1* gene that expresses all of its known mutants. Tests used for diagnosis include chromosome analysis and various protein tests. Diagnosis is usually made

when a child is young, and there is no current cure for this illness. Early diagnosis of the syndrome may allow for therapeutic interventions such as speech therapy, occupational therapy, psychotherapy, and special education, which can improve the quality of a patient's life considerably.

23-Hemophilia

It has been reported that the Arab physician Abu al-Qasim al-Zahrawi, commonly known as Abulcasis, wrote a book on hematology. His book *Al-Tasrif* written during the period 1000 AD provided the first description of hemophilia, a hereditary genetic disorder

Disorder and Genetics

Hemophilia is a hereditary bleeding disorder resulting from a partial or total lack of an essential blood clotting factor. It is a lifelong disorder that produces excessive bleeding, and spontaneous internal bleeding occurs very frequently. Hemophilia A, the most common form, is caused by a deficiency in clotting factor 8 (factor VIII). Hemophilia B results from a deficiency in clotting factor 9(IX). This disorder is inherited in a sex linked recessive manner.

Prevalence

This disorder occurs more prominently in males than in females. About one-third of new diagnoses occur without a family history. It appears globally and occurs in all racial groups.

Diagnosis and Prognosis

Hemophilia can be diagnosed as type A or type B by blood tests in infants after 9 months of age. Administration of clotting factors helps affected individuals to live with the disease. Hemophilia, though a serious disease, can be tolerable with proper precautions and therapy, and the prospects for children with hemophilia are excellent. Recent studies have documented a greatly increased quality of life and life expectancy among hemophiliac patients in developed countries.

However, it is predicted that the number of people with hemophilia in developed countries will increase gradually during the next few decades.

24-Huntington Disease

Disorder and Genetics

Huntington disease is an autosomal dominant genetic disorder; if one parent carries the defective Huntington disease gene, his or her offspring have a 50-50 chance of inheriting the disease. Huntington disease is a neurodegenerative brain disorder in which afflicted individuals lose their ability to walk, talk, think, and reason. They easily become depressed, lose their short-term memory, and may experience a lack of concentration and focus. Every individual with the gene for the disease eventually develops the disease.

The Huntington disease gene is located on the long arm of human chromosome 4 and encodes a protein called **huntingtin** that is quite variable in its structure. The 5' end of the Huntington disease gene contains many repeats of the CAG trinucleotide (encodes glutamine). A highly variable number of **CAG trinucleotide repeats** accounts for the Huntington disease gene mutation, which leads to the expression of an abnormally long **polyglutamine tract** at the N terminus of the huntingtin protein beginning at residue 18.

Such polyglutamine tracts increase protein aggregation, which may alter cell function. Thus, like fragile X syndrome, Huntington disease is one of 14 trinucleotide repeat disorders that cause neurological dysfunction in humans. More specifically, Huntington disease is classified as a **polyglutamine disorder**.

Healthy unaffected persons have a CAG repeat count of 9 to 35 (Table). However, alleles with more than 36 CAG repeats give rise to Huntington disease (the highest reported repeat length is 250). Incomplete penetrance is found in alleles with 36 to 39 CAG repeats. People with 36 to 40 CAG repeats may or may not develop Huntington disease, while people with more than 40 CAG repeats are rather likely to develop the disorder. Alleles with more than 60 CAG repeats result in a severe form of Huntington disease known as juvenile Huntington disease, and children who get Huntington disease may range from 2 to 20 years of age.

Table- Variation in status of Huntington disease is controlled by number of CAG repeats in the *huntingtin* gene

CAG repeat count	Disease classification	Disease status
<28	Normal	Unaffected
28–35	Intermediate	Unaffected
36–40	Incomplete penetrance	Weakly affected with Huntington disease
>40	Full penetrance	All affected with Huntington disease
>60	Full penetrance	Affected with juvenile Huntington disease

The mass of the huntingtin protein depends mainly on the number of its glutamine residues. Wild-type (normal) huntingtin consists of 3,144 amino acids, contains 6 to 35 glutamines, and has a mass of ~350 kilodaltons (kDa). In Huntington disease patients, huntingtin contains more than 36 glutamines and has an overall higher molecular mass than wild-type huntingtin.

Prevalence

Huntington disease affects males and females equally and crosses all ethnic and racial boundaries. Usually, Huntington disease begins at age 30 to 45, but it may occur as early as the age of 2. Children who develop juvenile Huntington disease rarely live to adulthood. Everyone who carries the gene develops the disease. In the United States, Canada, and western Europe, Huntington disease affects about 1 in 20,000 people.

Diagnosis and Prognosis

Currently, there is no treatment or cure for Huntington disease. The discovery of the Huntington disease gene in 1993 facilitated the development of specialized testing that may help to confirm the diagnosis of the disease in patients with an affected parent or characteristic symptoms of the disease. Blood samples are taken from patients, and DNA is directly analyzed by PCR for Huntington disease gene mutations to determine the number of CAG repeats in the Huntington disease gene region. Additional blood samples may be obtained from close or first-degree relatives (e.g., the mother or father) with Huntington disease to help confirm the results.

25-Sickle-Cell Anemia

Sickle-cell disease, or sickle-cell anemia or drepanocytosis, is a hereditary blood disorder, characterized by red blood cells that assume an abnormal, rigid, sickle shape. The sickling occurs because of a mutation in the hemoglobin gene.

Disorder

Sickle-cell disease is a blood-related disorder resulting from the substitution of a valine for glutamic acid in the β -globin chain of adult hemoglobin. The mutated sickle hemoglobin undergoes conformational change and polymerization upon deoxygenation, leading to red blood cell hemolysis and deformation (not doughnut shaped) and to pathology due to blockage of capillaries. Sickled red blood cells cannot migrate through small blood vessels; rather, they cluster and block these vessels, depriving organs and tissues of oxygen-carrying blood. This process leads to periodic episodes of pain and ultimately can damage tissues and vital organs and lead to other serious medical problems. Normal red blood cells live about 120 days in the bloodstream, but sickled red blood cells die much more rapidly, after about 10 to 20 days. Since they cannot be replaced fast enough, the blood is chronically short of red blood cells, leading to the condition of sickle-cell anemia.

Genetics

Hereditary persistence of fetal hemoglobin decreases the severity of sickle-cell disease. Thus, induction of fetal hemoglobin in adults has been a long-standing goal of therapies for sickle-cell disease. Recently, a GWAS for the fetal hemoglobin phenotype revealed a single strong locus, with sequence variants in the intron of a transcription factor, B-cell chronic lymphocytic leukemia/lymphoma 11A (BCL11A). BCL11A represses fetal hemoglobin expression in red blood cells.

Inactivation of BCL11A in a mouse model of sickle-cell disease not only leads to the induction of fetal hemoglobin expression but also corrects the hematologic and pathological defects of the disease. Thus, BCL11A is a potential therapeutic target for sickle-cell disease.

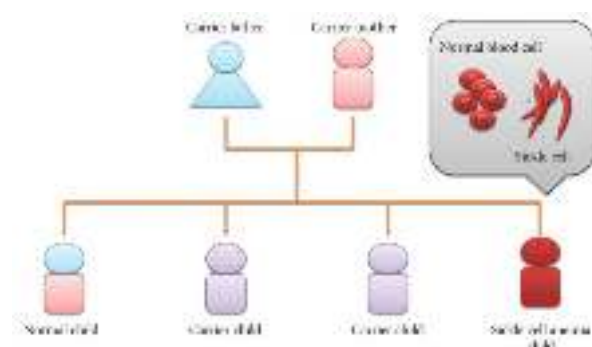


Figure- Single gene inheritance which causes sickle cell anemia

Prevalence

Sickle-cell anemia affects millions of people throughout the world. It is particularly common among people whose ancestors originate from sub-Saharan Africa, South America, Cuba,

Central America, Saudi Arabia, India, and Mediterranean countries (e.g., Turkey, Greece, and Italy).

In the United States, it affects around 72,000 people, most of whose ancestors come from Africa. The disease occurs in about 1 in every 500 African-Americans and 1 in every 1,000 to 1,400 Hispanic Americans. About 2 million Americans, or 1 in 12 African-Americans, carry the sickle-cell allele.

Diagnosis and Prognosis

Sickle-cell disease can be diagnosed by a simple blood test. In many cases, sickle-cell anemia is diagnosed when newborns are screened. Vaccines, antibiotics, and folic acid supplements are administered in addition to painkillers. Blood transfusions and surgery are used in severe cases. The only known cure at present is a bone marrow transplant.

26-Tay-Sachs Disease

Disorder and Genetics

Tay-Sachs disease is an autosomal recessive fatal genetic disorder caused by a genetic mutation in the *HEXA* gene on human chromosome 15, which encodes the hexosaminidase A enzyme. This mutation leads to a decrease in function of hexosaminidase A, and as a result, harmful quantities of a sphingoglycolipid, termed ganglioside GM2, accumulate in brain neurons.

HEXA gene mutations are rare and are most frequently detected in genetically isolated populations. Tay-Sachs disease can occur from the inheritance of either two similar or two unrelated mutations in *HEXA* that cause disease. Many *HEXA* mutations have been identified, and these mutations can reach significant frequencies in certain populations (see below).

In the most common form of the disease (infantile Tay-Sachs), abnormal hexosaminidase A enzyme activity and the accompanying harmful accumulation of cell membrane-associated gangliosides in neurons lead to premature neuronal death, paralysis, dementia, blindness, psychoses, and even death of the patient. Although the degeneration of the central nervous system begins at the fetal stage, the loss of peripheral vision and motor coordination are not evident until ~6 months of age, and death usually results by 4 years of age.

Prevalence

The frequency of Tay-Sachs disease is much higher in Ashkenazi Jews (Jews of eastern European origin) than in others. Approximately 1 in 27 Jews in the United States is a carrier of the Tay-Sachs disease gene. There is also a noticeable incidence of Tay-Sachs disease in non-Jewish French Canadians, known as Acadians, who originated from France and settled in southeastern Quebec. Interestingly, while the French Canadians and Ashkenazi Jews carry different *HEXA* mutations, the Cajuns (people in southern Louisiana descended from the Acadians) carry the mutation found most frequently in Ashkenazi Jews. The ancestry of carriers from Louisiana families traces back to a single non-Jewish founder couple that lived

in France in the 18th century. The Irish are also at increased risk for the Tay–Sachs gene, and among Irish-Americans the carrier rate is currently about 1 in 50. By contrast, the carrier rate in the general non-Jewish population as well as in Jews of Sephardic (Iberian or Middle Eastern) origin in the United States is about 1 in 250.

Diagnosis and Prognosis

Tay–Sachs disease may be diagnosed by a blood test that measures levels of the hexosaminidase A enzyme in serum, lymphocytes, or skin fibroblasts. During the past 25 years, carrier screening and genetic counseling in high-risk populations have greatly reduced the number of children born with Tay–Sachs disease in these groups. Thus, a high percentage of babies born with Tay–Sachs disease today are born to couples not previously considered to be at high risk. Prenatal tests of hexosaminidase A activity, such as amniocentesis (at 15 to 16 weeks of pregnancy) and chorionic villus sampling (at 10 to 12 weeks of pregnancy), can now diagnose Tay–Sachs disease in the fetus.

27-Thalassemia

Disorder and Genetics

Thalassemia is a blood-related genetic disorder that involves either the absence of or errors in genes responsible for the production of hemoglobin. Each red blood cell contains 240 million to 300 million molecules of hemoglobin. The severity of the disease depends on the gene mutations

that arise and the manner in which they influence each other.

Both of the hemoglobin α and β subunits are required to bind oxygen in the lungs and deliver it to other tissues. Genes on human chromosome 16 encode the α subunits, while genes on chromosome 11 encode the β subunits. A lack of expression of a particular subunit determines the type of thalassemia; e.g., a lack of an α subunit results in α -thalassemia. The lack of subunits corresponds to mutations in the genes on the appropriate chromosomes. There can be various grades of the disease depending on the gene and the type of mutations.

Prevalence

The α - and β -thalassemias are the most common inherited single-gene disorders, with the highest prevalence in areas where malaria was or still is endemic. The burden of this disorder in many regions is of such a magnitude that it represents a major public health concern. In Iran, about 8,000 fetuses may be affected by thalassemia each year. In some Mediterranean countries, control programs have achieved 80 to 100% prevention of disease in newborns.

Diagnosis and Prognosis

Diagnosis of thalassemia can be made as early as 10 to 11 weeks in pregnancy using procedures such as amniocentesis and chorionic villus sampling. Individuals can also be tested for thalassemia through routine blood counts. Thalassemic patients may have reduced

fertility or even infertility. Early treatment of thalassemia has been very effective in improving the quality of life of patients. Treatments for thalassemias depend on the type and severity of the disorder. Carriers or individuals who have mild or no symptoms of α - or β -thalassemia need little or no treatment.

Three types of treatments are used for moderate and severe forms of thalassemia. These include blood transfusions, iron chelation therapy (removes excess iron in the blood that increases after transfusions), and folic acid supplements (vitamin B supports red blood cell growth). Currently, genetic testing and counseling, and prenatal diagnosis, play an increasingly important role in informing individual as well as professional decisions related to the prevention, management, and treatment of this disease.

28-Polygenic disorders- Diabetes

Genetic disorders are **polygenic** if they are causally associated with the effects of many genes. Such disorders may also be **multifactorial**, if they are influenced by several different lifestyles and environmental factors.

Examples of multifactorial disorders include cancer, heart disease, and diabetes. Although such disorders frequently cluster in families, these disorders do not have a simple Mendelian pattern of inheritance. Moreover, as many of the factors that cause these disorders have not yet been identified, it is rather difficult to determine the risk of inheritance or transmission of these disorders

Some principal features of the pattern of multifactorial inheritance of a polygenic diseases are summarized in Table.

No.	Characteristic
1	A simple pattern of inheritance of a polygenic disease does not exist within a family.
2	A lower incidence of a polygenic disease within a population is associated with a higher risk of disease in first-degree relatives.
3	The relative risk of a polygenic disease is much lower in second-degree and more distantly related family members.
4	The risk of recurrence of a polygenic disease in a family is higher when more than one family member is affected.
5	The more severe the malformation associated with a polygenic disease, the greater is the risk of disease recurrence.
6	If a polygenic disease is more frequent in one sex than the other, the risk is higher for relatives of patients of the less susceptible sex.
7	If an increased risk of recurrence of disease occurs when the parents are consanguineous (e.g., first-degree relatives), many factors with additive effects may be involved.

An individual may not be born with a disease but may be at high risk of acquiring it, a condition referred to as **genetic predisposition** or **genetic susceptibility**. The genetic susceptibility to a particular disease due to the presence of a gene mutation(s) in an allele(s) need not lead to disease.

Diabetes is a disease in which the body does not produce or properly use insulin. Insulin is a hormone that is needed to convert sugar, starches, and other food into energy needed for daily

life. The cause of diabetes continues to be a mystery, although both genetics and environmental factors such as obesity and lack of exercise appear to play roles. There are three major classes of diabetes: type 1 diabetes, type 2 diabetes, and gestational diabetes. Only type 1 diabetes, a representative polygenic autoimmune disease.

29-Diabetes mellitus type 1

Disorder

Type 1 diabetes is an autoimmune disease that presents clinically with hyperglycemia resulting from the immune-mediated progressive destruction of insulin-producing pancreatic islet β -cells and associated metabolic dysfunction.

The resulting insulin deficiency requires lifelong exogenous insulin treatment for survival, and long-term complications can cause substantial disability and shorten life span. About 90 to 95% of the β -cell mass is already destroyed when symptoms of type 1 diabetes first appear, demonstrating that prediction and prevention are high priorities.

This disease affects about 1 in 300 individuals in North America. More than 20 million people worldwide (mostly children and young adults) are estimated to have type 1 diabetes.

Genetics

The etiology and pathogenesis of type 1 diabetes are largely accounted for by genetic predisposition. Within a pair of monozygotic twins (originating from one placenta), if one twin has type 1 diabetes, the probability of the other twin becoming a type 1 diabetic is about 40 to 50%. In pairs of dizygotic twins (originating from two placentas) in which one twin is type 1 diabetic, the probability of the other twin becoming a type 1 diabetic is only about 5 to 6%. This lower probability of susceptibility to type 1 diabetes is the same as that for unrelated children in the general population. These findings are consistent with the observed overall global increase in type 1 diabetes incidence during the last 20 to 30 years and strongly support the idea that environmental factors are also important in the control of susceptibility to type 1 diabetes.

The majority of genetic research to date has focused on the heritability that predisposes to islet autoimmunity and type 1 diabetes. The evidence indicates that type 1 diabetes is a polygenic, common, complex disease, with major susceptibility genes located in the *HLA* complex on human chromosome 6 and with other smaller effects found in non-*HLA* loci on other chromosomes. Recent advances in DNA technology, including high-throughput SNP typing and sequencing and GWASs, have advanced our understanding of the immune pathogenesis of type 1 diabetes.

Diagnosis

Type 1 diabetes is checked by analyzing the plasma glucose level and is diagnosed by fasting plasma glucose level at or above 126 mg/dL (considered as prediabetic conditions) and plasma glucose at or above 200 mg/dL (considered as diabetic condition).

To confirm type 1 diabetes, most physicians prefer to measure a fasting glucose level and according to the current definition, two fasting glucose measurements above 126 mg/dL are considered diagnostic for diabetes mellitus. Moreover, patients with fasting glucose levels from 100 to 125 mg/dL are considered to have impaired fasting glucose.

Furthermore, patients with plasma glucose at or above 140 mg/dL but not over 200 mg/dL, 2 h after a 75 g oral glucose load are considered to have impaired glucose tolerance. Interestingly, among these two prediabetic states, the latter in particular is a major risk factor for progression to full-blown diabetes mellitus as well as cardiovascular disease.

30-Diabetes mellitus type 2

It is known as Non Insulin dependent diabetes (NIDD) and is characterized by reducing response of body against insulin or relatively reduced insulin secretion by the beta cells. Type 2 diabetes is the most common type and starts appearing after the age of 30 years. It is due to the combination of sedentary lifestyle and genetic factors.

Obesity has been found to contribute to approximately 55% cases of the type 2 diabetes. However, because type 2 diabetes develops slowly, some people with high blood sugar experience no symptoms at all.

Treatment: Onset of type 2 diabetes can be delayed or prevented through proper nutrition and regular exercise. If the condition progresses, medications may be needed.

The synthesized insulins are used medically to treat patients with Type 1 diabetes mellitus, whereas patients with Type 2 diabetes mellitus are insulin resistant.

31-Alzheimer-diseases

Alzheimer's disease (AD), also known in the medical literature as Alzheimer disease, is the most common form of dementia. There is no cure for the disease, which worsens as it progresses, and eventually leads to death. It was first described by a German psychiatrist and neuropathologist Alois Alzheimer in 1906 and was named after him. Many different clinical features are associated with Alzheimer disease, including the inability to create new memories, the loss of short-term memory, and the inability to concentrate.

Disorder

Alzheimer disease is a devastating neurodegenerative disorder with a relentless progression. The pathogenesis of Alzheimer disease may be triggered by the accumulation of the amyloid β peptide ($A\beta$), which is due to overproduction of $A\beta$ and/or the failure of mechanisms to clear the peptide. $A\beta$ self-aggregates into oligomers of various sizes and forms diffuse and neuritic plaques in the parenchyma and blood vessels. $A\beta$ oligomers and plaques are potent mediators of $A\beta$ toxicity, block proteasome function, inhibit mitochondrial activity, alter

intracellular Ca^{2+} levels, and stimulate inflammatory processes. Loss of normal $\text{A}\beta$ function also contributes to neuronal dysfunction.

$\text{A}\beta$ interacts with the signaling pathways that regulate the phosphorylation of the microtubule-associated protein tau. Hyperphosphorylation of tau disrupts its normal function in regulating axonal transport and leads to the accumulation of neurofibrillary tangles and toxic species of soluble tau. Degradation of hyperphosphorylated tau by the proteasome is inhibited by the actions of $\text{A}\beta$. These two proteins and their associated signaling pathways therefore represent

important therapeutic targets for Alzheimer disease. Abnormalities in $\text{A}\beta$ and tau can be measured upon neuropathological examination, in cerebrospinal fluid or by positive emission tomography scans.

Genetics

Susceptibility to Alzheimer disease is genetically controlled, and about 13% of Alzheimer disease is inherited as an autosomal dominant disorder. **Late-onset familial Alzheimer**

disease is a complex polygenic disorder controlled by multiple susceptibility genes, and the strongest association is with the *APOE* $\epsilon 4$ allele at locus *AD2*.

Mutations in three genes—those for **amyloid** precursor protein (*APP*), presenilin-1 (*PSEN1*), and presenilin-2 (*PSEN2*)—account for less than 5% of all cases of Alzheimer disease and often cause early onset of the disease in individuals who are 60 years old or younger. Thus, despite considerable research effort, the root cause of Alzheimer disease in the vast majority of cases is unknown.

Prevalence

Alzheimer disease is the most common cause of dementia in North America and Europe, with an estimate of four million affected individuals in the United States. The prevalence of Alzheimer disease increases with age: about 10% of persons older than 70 have significant memory loss, and more than half of these individuals have Alzheimer disease. An estimated 25 to 45% of persons over age 85 have dementia.

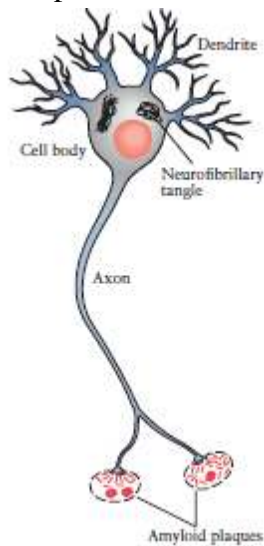
Diagnosis and Prognosis

A proper diagnosis of Alzheimer disease relies on a clinical neuropathological assessment. The formation of $\text{A}\beta$ plaques and neurofibrillary tangles is thought to contribute to the degradation of the neurons in the brain and the subsequent symptoms of Alzheimer disease. A hallmark of

Alzheimer disease is the accumulation of amyloid plaques between neurons in the brain. Amyloid is a general term for protein fragments that the body produces normally. $\text{A}\beta$ is a protein fragment cleaved from a larger amyloid precursor protein. In a healthy brain, these $\text{A}\beta$ protein fragments are broken down and eliminated. In Alzheimer disease, the fragments accumulate to form hard, insoluble plaques (deposits). The plaques are composed of a tangle of protein aggregates that have a fiber appearance and are called amyloid fibers.

Neurofibrillary tangles consist of these insoluble twisted amyloid fibers found inside neurons. These tangles are formed primarily by a protein called tau, which forms part of a microtubule that helps to transport nutrients and other substances from one part of a neuron to another. In

Alzheimer disease, however, the tau protein is abnormal and the microtubule structures collapse. As a result, neurons are deprived of certain nutrients and undergo cell death. In Alzheimer disease, however, the tau protein is abnormal and the microtubule structures collapse. As a result, neurons are deprived of certain nutrients and undergo cell death.



Neurofibrillary tangles and amyloid plaques associated with neurons in the brain are hallmarks of Alzheimer disease.

Three forms of early-onset familial Alzheimer disease caused by mutations in one of three genes—A β (A4) precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*)—are recognized. Molecular genetic testing of the three genes is available in clinical laboratories.

Early- onset familial Alzheimer disease A form of Alzheimer disease that arises at a younger age (<65 years) in families. About 1 to 6% of all Alzheimer disease is early onset, and about 60% of early- onset Alzheimer disease runs in families.

Late- onset familial Alzheimer disease A complex polygenic disorder controlled by multiple susceptibility genes. The strongest association is with the *APOE* e4 allele at locus **AD2**.

Senile plaque A dense spheroid amyloid body found outside of neurons and often associated with Alzheimer disease

32-Breast Cancer Disorder

Cancer generally results from the sequential acquisition of mutations in genes that regulate cell multiplication, cell repair, and the ability of a cell to undergo malignant transformation. This multistep process is not an abrupt transition from normal to malignant but may take 20 years or more.

Genetics

This malignancy is one of the most commonly inherited cancers based on observations that

- (i) 20 to 30% of all patients with breast cancer have a family history of the disease and
- (ii) Twin studies show that 25% of breast cancer cases are heritable.

Discovery of the breast cancer type 1 susceptibility (*BRCA1*) and *BRCA2* genes more than 10 years ago has had a high impact on patient care, allowing for early detection and prevention of breast cancer. However, deleterious mutations in the *BRCA1* and *BRCA2* genes cause at most 3 to 8% of all breast cancer cases.

Prevalence

Lung, stomach, liver, colon, and breast cancers cause the most cancer deaths each year. Combined, these cancers are responsible for more than 4.2 million deaths annually. Current epidemiological evidence predicts that 1 in 8 women will be diagnosed with breast cancer in her lifetime. Deaths from cancer worldwide are predicted to continue rising, with a projected 13.1 million deaths in 2030. Breast cancer is the second most common malignancy diagnosed in women and the second leading cause of cancer-related deaths.

Diagnosis and Prognosis

The roles that genes play differ greatly, ranging from completely determining the disease state (disease genes) to interacting with other genes and environmental factors in causing cancer (susceptibility genes). The primary determinants of most cancers are lifestyle factors (e.g., smoking, dietary and exercise habits, environmental carcinogens, and infectious agents) rather than inherited genetic factors.

Breast cancer is a heterogeneous disease often characterized by the presence or absence of expression of estrogen receptors and human epidermal growth factor receptor 2 (HER2) on tumor cells. These molecular markers used to classify breast cancer subtypes may also predict typical responses to targeted therapies.

Genetic tests will be useful in determining the best course of treatment for cancers, which are presently classified as a single disease but may ultimately be classified into different types, each best managed by a different therapeutic strategy.

Thus, genetics will continue to play an important role in the control of cancer, including

- (i) The identification of individuals at risk for a specific cancer, leading to preventative or screening strategies for an individual or family members, and
- (ii) The identification of the subtype of a cancer so that treatment can be tailored to target that specific disease.

Women with a type of breast cancer that overproduces the human epidermal growth factor receptor 2 (HER2) protein on the surface of the tumor may be effectively treated with the humanized anti-HER2 monoclonal antibody trastuzumab (Herceptin).

Trastuzumab: The First Humanized Monoclonal Antibody Approved for the Treatment of Breast Cancer.

33-Cardiovascular Disease

Disorder

Major cardiovascular diseases include several subtypes, such as coronary heart disease, cerebrovascular disease, heart failure, rheumatic heart disease, and congenital heart disease. Cardiovascular disease is a leading health problem, affecting more than 80 million individuals in the United States. In Canada, 1 out of 3 deaths is attributable to cardiovascular disease.

The major risk factors associated with cardiovascular diseases are cigarette smoking, unhealthy diet, physical inactivity, hypertension, diabetes, and high blood cholesterol. Cardiovascular diseases may also result from a variety of genetic causes, including single-gene mutations, interactions between multiple genes, and gene–environment interactions.

Economic shifts, urbanization, industrialization, and globalization bring about lifestyle changes that promote heart disease. In developing countries, life expectancy is increasing rapidly and is expected to expose people to these environmental risk factors for longer periods.

Genetics

For most cardiovascular disease disorders, the relative risk of disease is attributable to inherited DNA sequence variants. However, the role of inheritance and the magnitude of its effect vary by disease and other factors such as age of disease onset and subtype of cardiovascular disease. Most cardiovascular disease traits, such as myocardial infarction and concentrations of plasma LDL cholesterol, show complex inheritance, suggestive of an interaction between multiple genes and environmental factors.

Disease	Possible causes	Affects	Symptoms	Treatment
Cardiovascular disease	Inherited and acquired, predominant lifestyle disease caused by smoking	Coronary arteries are at risk for narrowing as cholesterol deposits, called plaques, build up inside the artery, affect the heart muscle or the blood vessels of the heart which can lead to congestive heart failure, and may also cause disturbances in normal heartbeat, called arrhythmia; clot in artery due to rupture of plaque can lead to myocardial infarction	Chest pain, shortness of breath, sweating, nausea, and indigestion	Medical intervention with multistep approach, lifestyle modification, regular exercise, cholesterol reduction, quitting smoking and alcohol consumption, and stress-free life
	High blood pressure (hypertension)			
	High cholesterol			
	Diabetes			
	Family history			
	Peripheral artery disease			
	Obesity			

34-Mitochondrial Disorders

Disorders

For cells to function normally, they must generate energy in the form of ATP. In many types of cells, mitochondrial activity is the prime source of ATP. Mitochondria also regulate

several other cellular processes, including production of heat in response to changes in temperature and diet, ion homeostasis (maintenance of an internal steady state of ions), innate immune response, production of reactive oxygen species, and programmed cell death (apoptosis).

It follows that if mitochondrial malfunction occurs and leads to deficient energy production and the impairment of other cellular functions, this may result in a mitochondrial disease(s). The knowledge that mitochondrial impairment may be involved in diseases is relatively new; it was first recognized in an adult in the late 1960s and then in children in the late 1980s.

Mitochondrial dysfunction can occur in the cells of many organs and systems in the body; more than 200 inherited diseases of metabolism can affect mitochondria, and more than 40 types of mitochondrial disorders have been reported. The brain, heart, muscles, and lungs are the organs that require the most energy, and when deprived of energy the functions of these organs begin to fail.

The most severe effects of mitochondrial disease occur in the brain and muscles because they are heaviest users of energy. Other commonly affected organs include the liver, nervous system, eyes, ears, and kidneys. Mitochondrial dysfunction is observed in single-gene mitochondrial disorders and may also be associated with the pathogenesis of polygenic diseases such as Alzheimer disease, Parkinson disease, cancer, cardiac disease, diabetes, epilepsy, Huntington disease, and obesity. In addition, a progressive decline in the expression of mitochondrial genes is a main feature of human aging, raising the possibility that an increased understanding of the aging process may provide more insight into one or more of the above-mentioned polygenic diseases.

Genetics

Mitochondrial dysfunction generally results from a genetic mutation(s). Interestingly, mitochondria have their own DNA for 37 genes, 13 mitochondrial proteins, 2 rRNAs, and 22 tRNAs. Mitochondrial DNA is less complex than nuclear DNA and is not inherited according to Mendelian genetics (inheritance of DNA from both parents). Rather, because egg cells but not sperm contribute mitochondria to a developing embryo, only mothers can transmit mitochondrial traits to their children. Thus, mitochondrial DNA is inherited only maternally.

Mutations in mitochondrial DNA differ from those in nuclear DNA, and many more mutations occur in mitochondrial DNA than nuclear DNA. The mutation rate in mitochondrial DNA is 10 times higher than in nuclear DNA because mitochondrial DNA is subject to damage from reactive oxygen molecules released as a by-product during oxidative phosphorylation in cells. Mitochondrial dysfunction may also be caused by environmental factors (e.g., viral infection or drug treatment) that interfere with mitochondrial activity.

35-Mitochondrial homeostasis

Because neurons require considerable energy to maintain their metabolically active state, they rely heavily on the maintenance of mitochondria in a functional state, a property known as **mitochondrial homeostasis**.

Mitochondria constitute a population of organelles that require a careful balance and integration of many cellular processes, including the regulation of biogenesis, migration throughout the cell, shape remodeling, and autophagy (catabolic degradation of unnecessary cellular components by lysosomes).

These dynamic processes prompt mitochondrial recruitment to critical subcellular compartments, content exchange between mitochondria, mitochondrial shape control, and mitochondrial communication with the cytosol. Mitochondrial homeostasis is the term used to refer to a well maintained balance of these processes. The structure and function of the **mitochondrial network are dependent on mitochondrial homeostasis, which is essential for** maintaining the signaling, plasticity, and transmitter release of neurons. There are different pathways to maintain mitochondrial homeostasis.

36-Parkinson Disease

After Alzheimer disease, Parkinson disease is the second most common neurodegenerative disease. Parkinson disease affects about 1% of the population that is 60 years of age or older and is characterized by the progressive reduced capacity to initiate voluntary movements arising mainly from the loss of neurons that synthesize the neurotransmitter dopamine.

Among the many factors that mediate the development of Parkinson disease, mitochondrial dysfunction is considered to be a major factor in its etiology and pathogenesis. The dysfunction of mitochondria in Parkinson disease patients may result from one or more of the following conditions:

- Deletion of mitochondrial DNA,
- Accumulation of mitochondrial DNA
- Mutations, increase in oxidative stress from reactive oxygen species
- Deficient expression and function of the mitochondrial respiratory chain
- Abnormal morphology of mitochondria.

Neurodegenerative diseases such as Parkinson disease are often closely associated with an imbalance of mitochondrial homeostasis (Fig.). The levels of many mitochondrial proteins are altered in postmortem samples of brains from persons with Parkinson disease.

Genetic mutations in the *PTEN-induced putative kinase 1 (PINK1)*, *parkin (PARK2)*, *DJ-1*, *alpha-synuclein (SNCA)*, and *leucine-rich repeat kinase 2 (LRRK2)* genes are closely linked to the recessive Parkinson disease genes. Moreover, these mutations are associated with important functions that maintain mitochondrial homeostasis, which include membrane potential, calcium homeostasis, structure of cristae (internal compartments formed by the

inner membrane of mitochondria), respiratory activity, mitochondrial DNA integrity, and clearance of dysfunctional mitochondria from cells.

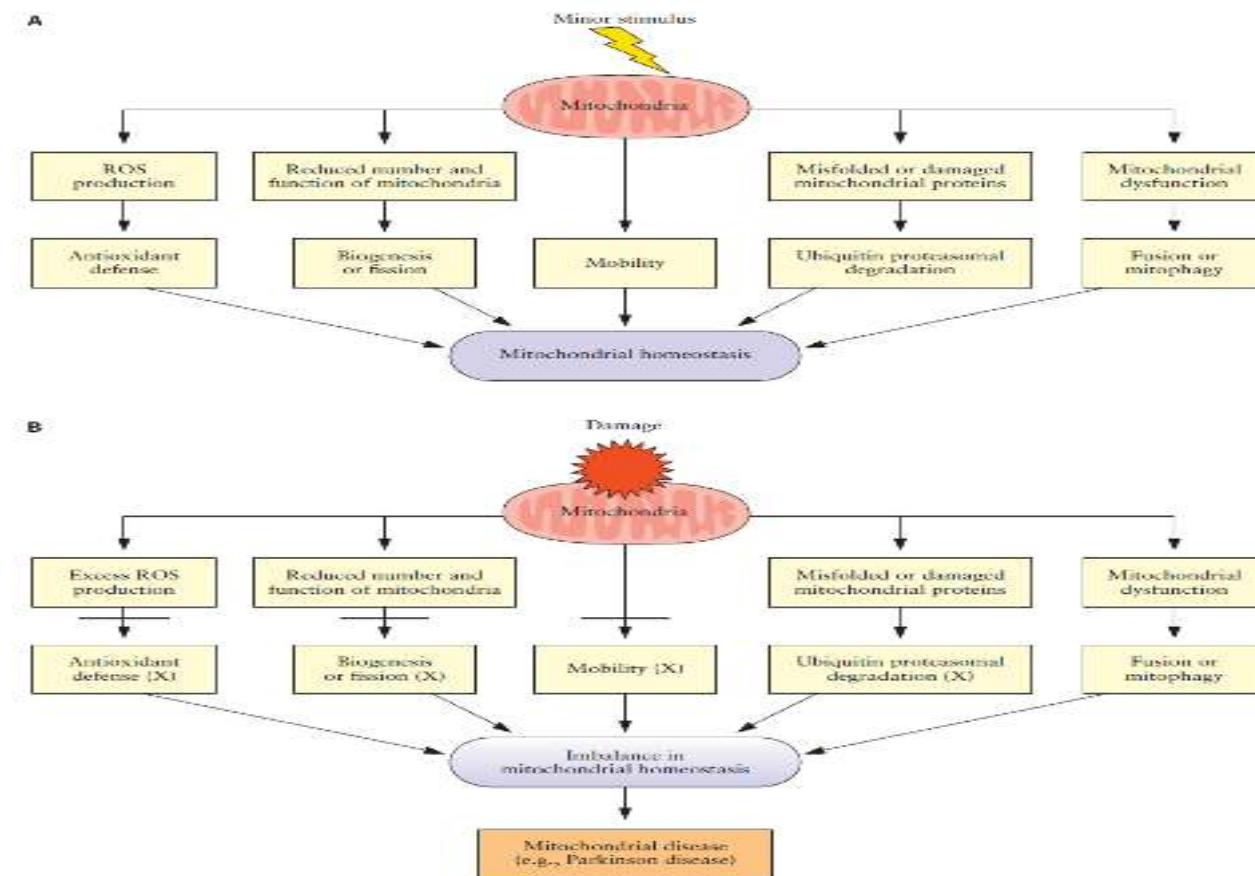


Fig-Parkinson disease is associated with an imbalance in mitochondrial homeostasis. Since mitochondria regulate energy metabolism, they are essential for the control of neuronal function. Mitochondria respond to exogenous and endogenous stimuli and maintain their homeostasis by undergoing continuous fusion, fission, mobility, mitophagy, and biogenesis. **(A)** Normally, upon exposure of mitochondria to a minor stimulus, changes in mitochondrial function and morphology occur to activate different cellular pathways that help to maintain homeostasis. **(B)** After mitochondria incur damage, changes in mitochondria occur that lead to excess reactive oxygen species (ROS) production, reduced number and mass, misfolding and/or aggregation of damaged proteins, and mitochondrial dysfunction. As a result, an imbalance in mitochondrial homeostasis ensues that may lead to the onset of Parkinson disease. Mitophagy is a catabolic process of self-degradation of mitochondria in a cell. X indicates a mitochondrial dysfunction.

37-Immunological Approaches To Detect Protein Biomarkers of Disease

Proteins play a critical role in all cellular processes, including metabolism, communication, defense, reproduction, transport, and motility. Regulatory proteins maintain tight control over protein production to ensure that cells function normally.

Characteristic changes in proteins have been used extensively to diagnose disease, either by detecting the presence or measuring levels of a specific protein biomarker or by determining

protein profiles in polygenic diseases. There are several advantages to using proteins as diagnostic biomarkers of disease. Abnormal levels of gene expression as a consequence of disease-associated mutations are more accurately quantified by measuring the protein directly rather than by measuring mRNA levels, which often do not correlate with protein levels.

Furthermore, proteins in diseased tissues may exhibit irregularities in posttranslational modification that cannot be detected using nucleic acids. In addition, many diseases often are a consequence of altered protein conformation (e.g., prion diseases and some neurodegenerative diseases) that may not be detected from nucleic acid sequences. On the other hand, the unique shape and often very low cellular levels of a protein are a challenge for development of a diagnostic assay. Antibodies can be produced that bind to target proteins with high affinity and specificity, and therefore immunological approaches meet the criteria of sensitivity, specificity, and simplicity for diagnostic assays.

Agglutination is a simple, inexpensive, rapid, and highly specific immunological test that is widely performed in diagnostic laboratories. For example, it is often used for human blood typing based on the presence of specific antigens on the surface of red blood cells, which vary among individuals.

Antiserum containing antibodies against either A or B surface antigen is mixed with red blood cells. Clumping (agglutination) indicates the presence of the antigen (Fig. A). Some individuals produce only the A antigen (blood type A), which agglutinates with anti-A antiserum but not with anti-B antiserum (Fig. A).

Blood type B individuals produce only the B antigen, which reacts with anti-B antiserum and not with anti-A antiserum (Fig. B). Others carry both antigens (blood type AB) or neither (blood type O). Blood samples from individuals with blood type O, the most common blood type, do not produce a positive agglutination result with either antiserum. This test, referred to as hemagglutination, is important for determining which blood to use in transfusions, as antibodies naturally produced against nonself red blood cell antigens would destroy the introduced blood. In some agglutination tests, the antigen or antibody employed to detect either a specific antibody or antigen, respectively, in patient samples is used to coat the surface of small latex beads.

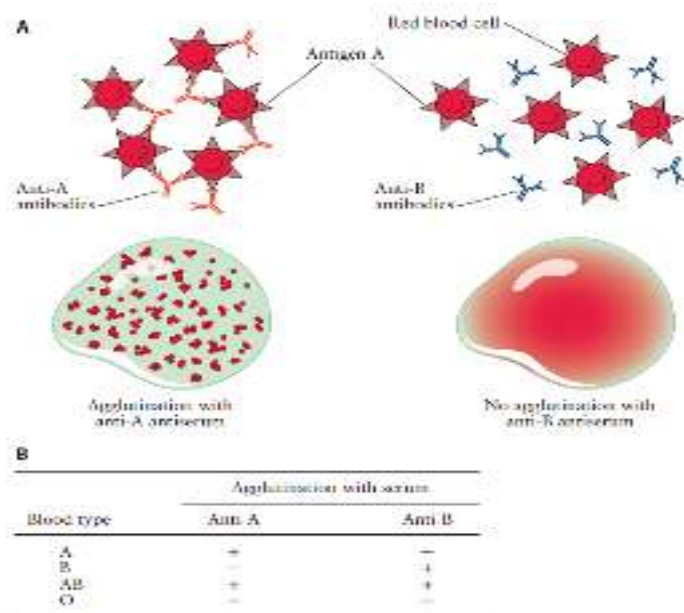


Fig-Hemagglutination test to determine blood type. **(A)** Antiserum containing antibodies against A or B surface antigens is mixed with red blood cells carrying A surface antigens. Clumping (agglutination) in the presence of anti-A antiserum but not with anti-B antiserum indicates blood type A. **(B)** Agglutination responses for different blood types.

38-Enzyme-Linked Immunosorbent Assays

Enzyme-linked immunosorbent assays (ELISAs) are widely used for diagnosing human diseases, including various cancers, autoimmune diseases, allergies, and infectious diseases. An ELISA measures antigens or antibodies produced against an antigen in a clinical sample from blood, urine, or tissues. It is based on the specific and high-affinity interaction between an antibody and an antigen and is a sensitive assay that can be used for rapid detection on a large scale (i.e., high-throughput assays). Detection relies on the activity of an enzyme that is covalently bound to an antibody employed in the assay.

- An **indirect ELISA** can detect the presence of specific antibodies in patient serum that indicates an immune response to the presence of a particular protein or pathogen.
- A **sandwich ELISA** detects the presence of a specific antigen in a patient's sample and hence is sometimes referred to as an antigen capture assay.

In an indirect ELISA used for diagnostic purposes, a standardized antigen is bound to a solid support, usually the surface of a well in a microtiter plate (Fig. A). A patient's serum sample is applied to the well, and specific **primary antibodies** in the serum bind to the immobilized antigens. A **secondary antibody** that binds specifically to the primary antibody is applied. Because the primary antibody is present in human serum, the secondary antibody is an anti-human immunoglobulin antibody that was raised in another animal, for example, a goat, by injecting the animal with human immunoglobulin. The secondary antibody is covalently

bound (conjugated) to an enzyme such as alkaline phosphatase or horseradish peroxidase that catalyzes the conversion of a colorless substrate into a colored product. A colorless substrate is applied, and the formation of a colored product by the enzyme indicates the presence of the secondary antibody and, hence, the primary antibody–antigen complex.

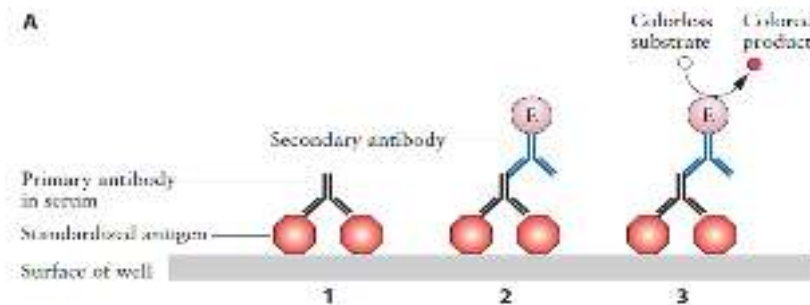


Fig. (A) Indirect ELISA. A specific antigen is immobilized on the surface of the wells in a microtiter plate. (1) Patient serum is applied, and primary antibodies present in the serum bind to the antigen. Unbound antibodies are removed by a washing step. (2) A secondary antibody that binds specifically to the primary antibody is applied. (3) The secondary antibody is conjugated to an enzyme (E) that catalyzes the conversion of a colorless substrate into a colored product. The formation of a colored product indicates that the serum sample contains antibodies directed against the antigen.

In contrast to an indirect ELISA, a sandwich ELISA directly detects a particular antigen in a complex clinical sample. To capture the antigen, a **monoclonal antibody** that is specific for the target antigen is first bound to the surface of a microtiter plate (Fig. B). The patient's sample is then added to a well; if the specific antigen is present in the sample, it binds to the immobilized antibody. A labeled primary antibody is added to detect the presence of bound antigen. The labeled antibody may detect a different **epitope** on the same antigen.

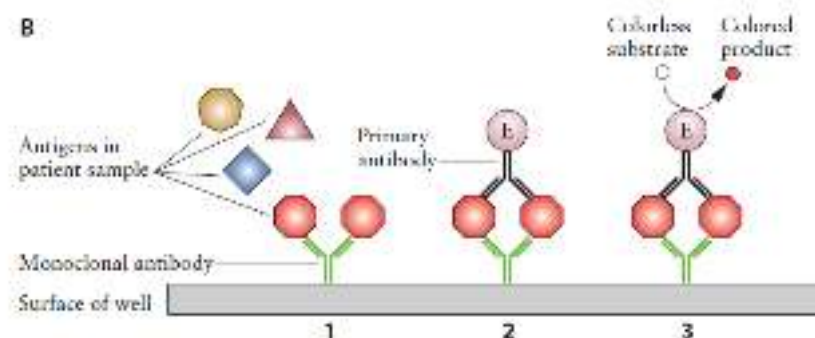


Fig. (B) Sandwich ELISA. Monoclonal antibodies specific for a target antigen are bound to the surface of a well in a microtiter plate. (1) A patient's sample is added to the wells, and specific antigens present in the sample bind to the immobilized antibody. A wash step removes any unbound molecules. (2) A primary antibody is applied to detect the presence of bound antigen. (3) The primary antibody is conjugated to an enzyme that catalyzes the formation of a colored product that indicates the presence of the target antigen.

39-Measuring Disease-Associated Proteins by Sandwich ELISA

Ovarian cancer is a devastating disease that kills over 15,000 women a year in the United States. More than 22,000 new cases are diagnosed each year, most at an advanced stage when the survival rate is less than 20%. An immunoassay widely used to monitor progression and recurrence of ovarian carcinoma measures levels of the protein CA125 in serum. CA125 is a high-molecular-weight glycoprotein that is present at higher levels in 50% of women with ovarian cancer compared to healthy women.

Levels may also be elevated in women with lung, pancreatic, breast, cervical, and colorectal cancers and with noncancerous disorders such as pelvic inflammatory disease, hepatic disorders, and nonmalignant ovarian cysts. Because CA125 may indicate other disorders and is not elevated in all patients with ovarian cancer, especially at early stages of the disease when tumors are small and therefore secrete only low levels of the protein, it is not recommended as a screening test. Rather, it is commonly used to monitor a patient's response to treatment. Researchers have therefore sought to identify other biomarkers that are more specific for ovarian cancer.

A useful biomarker for ovarian cancer is a protein that is secreted specifically by ovarian tumors, and not by normal tissue or other tumor types, and that can be detected at low levels in the blood or urine, enabling early, and noninvasive, detection. Using cDNA microarrays and quantitative polymerase chain reaction (PCR), human epididymis protein 4 (HE4) was identified as a promising new biomarker that was expressed in ovarian carcinomas but not normal tissue or benign ovarian tumors. Mouse monoclonal antibodies were generated against two different HE4 epitopes and used to develop an ELISA. In an initial blinded study (researchers were not informed of the source of the patient samples), HE4 was found to be elevated in sera from patients with early and late-stage ovarian cancer compared to sera from healthy women and from women with benign ovarian tumors.

The U.S. Food and Drug Administration (FDA) has approved the use of a HE4 sandwich ELISA to monitor the recurrence or progression of ovarian cancer in women who are being treated for the disease. Patient samples are incubated with biotinylated monoclonal antibodies generated against one HE4 epitope in streptavidin-coated wells (Fig.). A second antibody, conjugated to the enzyme horseradish peroxidase, that binds to a different HE4 epitope is added, and after generation of a colored product by the horseradish peroxidase, HE4 is quantified spectrophotometrically. Using this assay, HE4 was found to be present at elevated levels in more than 75% of patients with ovarian cancer, compared to 5% of healthy women and 13% of individuals with other nonmalignant conditions. An increase in HE4 levels of more than 25% is considered significant to suggest recurrence or disease progression, while a decrease of this magnitude suggests a positive response to treatment.

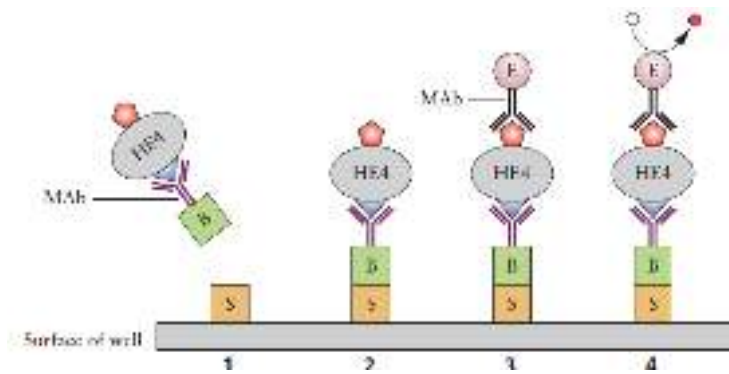


Fig- Sandwich ELISA to monitor progression or recurrence of ovarian cancer. **(1)** Patient samples are incubated with biotinylated (B) monoclonal antibodies (purple MAb) generated against one HE4 epitope (blue). **(2)** Biotin, together with the MAb–HE4 complex, binds with high affinity to streptavidin (S) that coats the wells of a microtiter plate. **(3)** A second antibody (black MAb) that binds to a different HE4 epitope (red) is added, and after generation of a colored product by horseradish peroxidase (E) conjugated to the antibody **(4)**, HE4 is quantified spectrophotometrically.

40-Diagnosing Autoimmune Diseases by an Indirect ELISA

Autoimmune diseases occur when the body's immune system does not recognize normal cellular molecules and structures as “self” but rather produces antibodies (autoantibodies) that destroy those targets. Several autoimmune diseases have been identified, including celiac disease, type 1 diabetes, lupus erythematosus, and rheumatoid arthritis.

Indirect ELISAs have been developed to diagnose some autoimmune diseases. In these assays, autoantibodies produced against a self-protein are detected in patient blood samples. Rheumatoid arthritis is an autoimmune disease that results in chronic, systemic inflammation of the joints, mainly the synovial (flexible) joints. The synovial membrane that lines these joints secretes a fluid that lubricates the articulating bones. Initially, an inflammatory response occurs in the synovial membranes in the small joints of the hands and feet; it then occurs in larger joints, causing accumulation of excess fluid and damage to the joints. Systemic inflammation may damage organs such as the heart and lungs. The disease affects about 1% of the population of the United States.

Rheumatoid factor is an autoantibody that targets the Fc region of immunoglobulin G (IgG) antibodies and contributes to rheumatoid arthritis. It is commonly used as a diagnostic biomarker to differentiate rheumatoid arthritis from other forms of arthritis and other inflammatory conditions. An indirect ELISA has been developed to detect the presence of rheumatoid factor in patient blood samples. In this test, IgG molecules, usually from a rabbit, are the standardized antigen bound to the surface of a multiwell plate, and diluted patient sera are applied to the wells. After incubation to allow binding of rheumatoid factor in the serum to the rabbit IgG molecules and washing to remove unbound molecules, an anti-human IgG antibody is added. The anti-human IgG antibody specifically targets rheumatoid factor (and

not rabbit IgG) and is conjugated to an enzyme such as horseradish peroxidase for colorimetric detection. Other ELISAs have been developed that target rheumatoid factor IgM and IgA that can assist in accurate diagnosis of rheumatoid arthritis.

Early diagnosis is important to prevent irreversible joint damage, as rheumatoid arthritis may be managed in the early stages by administering antirheumatic drugs.

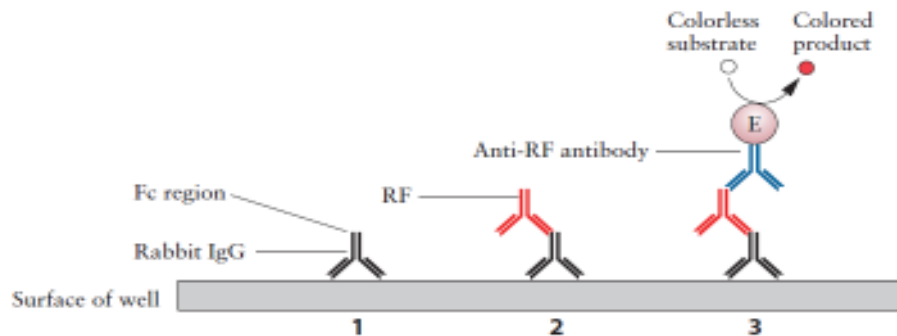


Fig- Indirect ELISA for diagnosis of the autoimmune disease rheumatoid arthritis. **(1)** Rabbit IgG molecules are bound to the surface of a microtiter plate, and diluted patient serum is applied. **(2)** If rheumatoid factor (RF) is present in the serum, it binds to the Fc region of the rabbit IgG molecules. **(3)** After a washing step to remove unbound molecules, an anti-RF antibody conjugated to an enzyme (E) is applied for colorimetric detection.

41-Immunoassay for infectious diseases

Clinical laboratories often identify pathogenic microbes in patient samples based on their physiological or biochemical characteristics. For example, a pathogenic bacterium may ferment specific carbohydrates or produce specific enzymes, and detection of the products of these reactions is the basis for some diagnostic assays. While these methods are effective, they require growth and isolation of the pathogen and therefore are slower than immunological and other molecular methods, typically requiring more than 48 h.

Immunological detection methods such as agglutination assays and ELISAs eliminate the need to grow the pathogen in culture and can be used to detect specific viral, bacterial, fungal, or protozoan pathogens in body fluids and tissues. Because antibody-based approaches detect a target antigen with high specificity and sensitivity, they are well suited to distinguish a specific pathogen from the other hundreds of microbes that are normally present in some human tissues. Immunological assays for infectious disease may target proteins produced by a pathogen or may detect the presence of antibodies produced against the pathogen. The latter, however, does not differentiate between current and past infections.

To prevent transmission of infectious disease, donated blood is screened for several pathogens, including human immunodeficiency virus and hepatitis viruses. Although five hepatitis viruses can infect the liver and cause liver inflammation, cell death, and even liver failure, most cases of chronic viral hepatitis are caused by hepatitis B and C viruses. Both viruses may be transmitted via blood transfusion.

ELISAs have been developed for routine blood screening for hepatitis B surface antigen, a viral envelope lipoprotein, and for antibodies produced against both hepatitis B and C viruses. Viral surface antigens are the first markers to be detected in serum after infection, while antibodies appear up to several months later. Detection of the viral surface antigen with a sandwich ELISA utilizes specific monoclonal antibodies to capture the hepatitis B surface antigens present in blood in wells of an assay plate (Fig. A). The monoclonal antibodies were generated using recombinant hepatitis B surface protein expressed in yeast. An anti-hepatitis B surface antigen detection antibody with a conjugated enzyme and the enzyme substrate are added to measure virus titers.

An indirect ELISA that detects the presence of anti-hepatitis B virus antibodies in the blood sample is often used to confirm a positive result (Fig. B); however, blood from individuals immunized with a hepatitis B vaccine also produces a positive result with this test. Blood may additionally be screened for hepatitis B DNA and C virus RNA.

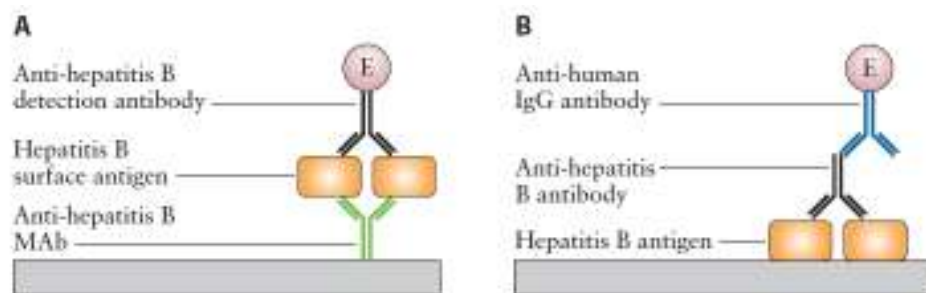


Fig- Diagnosis of hepatitis B virus infection by ELISA. **(A)** A sandwich ELISA detects the presence of hepatitis B surface antigen in blood. **(B)** An indirect ELISA detects the presence of anti-hepatitis B virus antibodies in blood.

42-Protein Array to Detect Polygenic Disease

An ELISA typically measures a single target protein; however, for some diagnoses, it may be more informative to measure multiple target proteins in a single assay. Analysis of **proteomes** is commonly used in research to identify and quantify protein changes in diseased tissue versus normal tissue and is useful for diagnosis of **polygenic diseases** (resulting from mutations in more than one gene) such as breast cancer, Alzheimer disease, type 1 diabetes, and cardiovascular disease.

Protein microarrays are multiplex immunoassays that can detect multiple biomarkers in a clinical sample. Biomarkers may be subsets of proteins in complex clinical samples such as biopsied tumor tissue. Currently, commercially available protein microarrays are most commonly used to detect antibodies in serum from patients suffering from allergies, autoimmune diseases, or infections.

Approximately 25% of the population in industrialized countries suffers from type I allergies, which are IgE-mediated immediate hypersensitivity reactions, such as asthma, hay fever, and eczema. In susceptible individuals, the first exposure to an allergen elicits the production of high levels of IgE antibodies that bind to mast cells. In this way, the individual becomes sensitized to the allergen. On subsequent exposures, the allergen reacts with mast cell-bound IgE and causes bridging between adjacent IgE molecules, thereby stimulating the release of inflammatory mediators such as histamine, leukotrienes, prostaglandins, and tumor necrosis factor. These biochemicals stimulate smooth muscle contraction and dilation of capillaries that cause edema (swelling), itching, and development of a rash.

Diagnostic allergen microarrays detect IgEs produced against common allergens, for example, pollen, food proteins, and molds. Purified allergens are arrayed in triplicate on a solid support and probed with sera from allergic patients (Fig.). Specific IgEs present in the serum bind to an immobilized allergen and are retained on the surface of the array. Bound IgEs are detected using an anti-human IgE antibody, often labeled with a fluorescent dye. The array format enables detection of hundreds of allergens in a single assay.

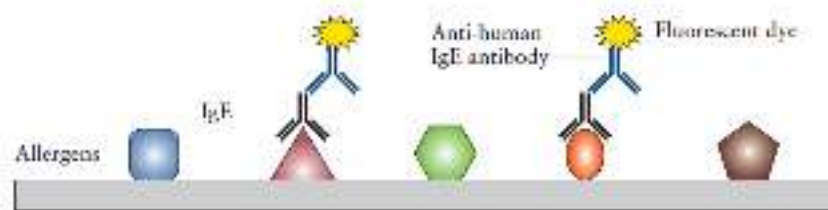


Fig- Diagnostic allergen microarray. Purified allergens are arrayed on a solid support and probed with serum from an allergic patient. Specific IgE present in the serum binds to an immobilized allergen and is detected using an anti human IgE antibody conjugated to a fluorescent dye

Another type of protein microarray measures levels of fifty different protein biomarkers of heart disease in a patient serum sample. Increased or decreased levels of these proteins have been associated with increased risk of heart disease. A liquid bead array has been developed to quantify levels of the different protein biomarkers are detected in a single well of a multiwell plate (Fig.), which enables rapid analysis of many patient samples simultaneously.

Primary antibodies directed against each protein biomarker are attached to tiny capture beads that are distinguished by either size, color, or shape. A serum sample is applied to a well, and specific proteins in the sample bind to their cognate antibody (Fig. A). For detection of the bound proteins, secondary antibodies conjugated to a fluorescent molecule are added to the well (Fig. B). Each bead, and any captured proteins, is analyzed by passing by a laser that measures fluorescence emitted from bound secondary antibodies and distinguishes the different capture beads by their size, color, or shape.

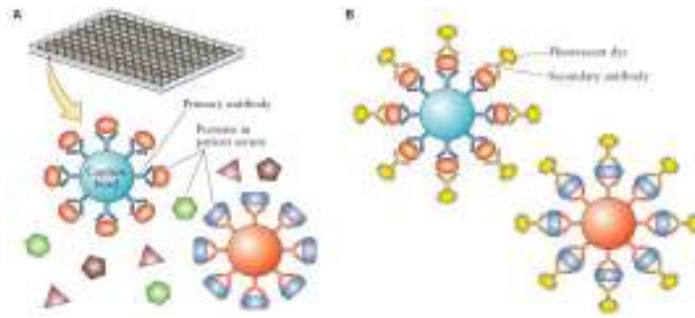


Fig- Liquid bead assay to detect protein biomarkers associated with increased risk of cardiovascular disease. **(A)** Patient serum is applied to a well of a microtiter plate containing capture beads bound to primary antibodies directed against all of the protein biomarkers. Capture beads of different colors distinguish primary antibodies that bind to different protein biomarkers. Target proteins in the sample bind to their cognate antibodies. **(B)** For detection of the bound proteins, secondary antibodies conjugated to a fluorescent molecule are added to the well. Each bead, and any captured proteins, is analyzed by passing by a laser that measures fluorescence. The presence of specific protein biomarkers is determined by the color of the capture bead to which they are bound.

43-Immunoassay for Protein Conformation-specific Disorder

Several human neurological disorders arise as a consequence of protein misfolding that leads to protein aggregation and cell death. Parkinson disease, Alzheimer disease, and prion diseases are examples of protein conformational disorders.

Alzheimer disease is a degenerative brain disorder that is characterized by the progressive loss of abstract thinking and memory, personality change, language disturbances, and a slowing of physical capabilities. Clinical diagnosis of Alzheimer disease is poor, although 1% of the population between 60 and 65 years old and 30% of the population over 80 years old may develop it. Two hallmarks of Alzheimer disease found in the brain are (i) neurofibrillary tangles of the cytoskeletal protein tau that accumulate within nerve cell bodies and (ii) dense extracellular aggregates of insoluble proteins called amyloid plaques that develop at the ends of inflamed nerves (Fig.).

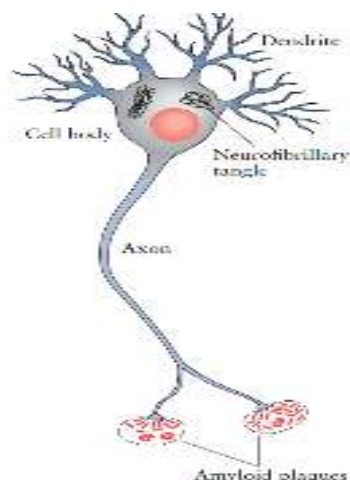


Fig- Neurofibrillary tangles and amyloid plaques associated with neurons in the brain are hallmarks of Alzheimer disease.

The principal protein of an amyloid plaque is a small protein called A β (β -amyloid protein). The A β protein ranges in length from 39 to 42 amino acid residues; the A β 40 and A β 42 forms are the main variants. All A β proteins are derived from the β -amyloid precursor protein (APP) by proteolytic cleavage. Abnormal cleavage of APP results in production of A β 40 and A β 42 and alters protein folding, causing exposed regions of the protein to self-interact; hence, the proteins aggregate in amyloid plaques.

Diagnosis of Alzheimer disease using immunological methods may exploit the development of antibodies that differentiate between the conformations of the disease-associated A β proteins and normal APP. To develop conformation-specific antibodies, researchers used an approach that mimics protein aggregation during the disease process, that is, the self-interaction of the A β proteins. They grafted short motifs (10-mers) from the A β 42 protein into a complementarity-determining region (CDR3, VH domain) of an antibody to generate antibodies that would interact with aggregated A β 42 via the grafted motif.

Out of twelve antibodies, carrying different but overlapping A β 42 sequences, three bound specifically to insoluble A β 42 aggregates but not A β 42 monomers. A motif of four amino acids, common to all three positive antibodies, was found to be the minimal sequence required for binding. To determine whether binding of the grafted antibodies is mediated by interactions between the A β motif on the antibody and the same motif in the aggregated A β proteins, each grafted antibody was bound to immobilized A β 42 aggregates and then a second antibody was applied. Binding of the second antibody was reduced only when it recognized a motif that overlapped with that grafted on the first antibody. This relatively simple approach may be used to generate conformation-specific antibodies against other proteins that not only aid in accurate disease diagnosis but also may be used in therapeutic strategies to target these proteins.

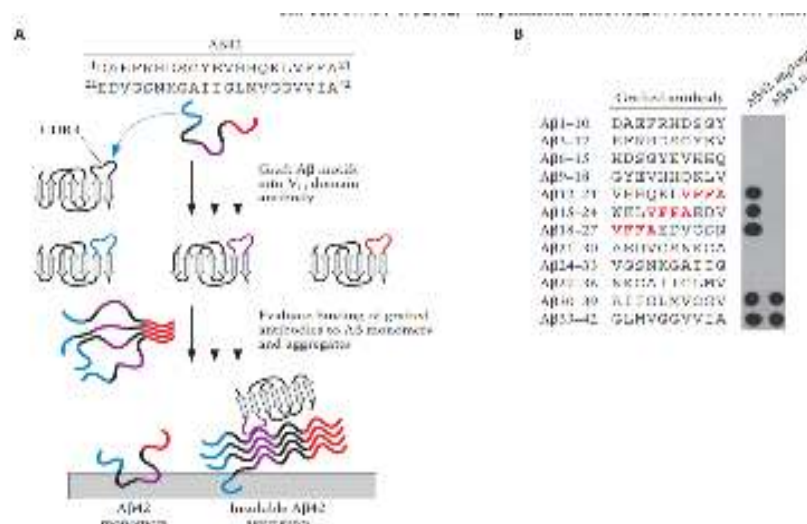


Fig- Development of conformation-specific antibodies to detect aggregated A β proteins indicative of Alzheimer disease. **(A)** Short (10-amino-acid) overlapping A β 42 peptide segments were grafted into a complementarity-determining region (CDR3, VH domain) to generate antibodies that bind specifically to aggregated, insoluble A β 42 proteins. **(B)** Out of 12 grafted antibodies, three (A β 12-21, A β 15-24, and A β 18-27) bound to aggregated

A β 42 proteins, and not A β 42 monomers, that were deposited on a membrane. A sequence of 4 amino acids (highlighted in red) was found to be the minimal requirement for binding.

44-DNA based approaches to diagnosis diseases

DNA-based diagnostic tests determine the existence of specific nucleotide sequences, including human genetic mutations and sequences present in human pathogens. They are highly sensitive and specific and can detect single nucleotide mutations or copy number variations. The ability to diagnose diseases in humans at the genetic level makes it possible to determine the cause of an illness and to predict whether individuals or their offspring are predisposed to the disease.

Because a DNA-based test does not require expression of a gene, in contrast to diagnostic detection of proteins, DNA analysis can be used for the identification of asymptomatic carriers of hereditary disorders, for prenatal diagnosis of serious genetic conditions, and for early diagnosis before the onset of symptoms. DNA sequence-specific diagnostic approaches include hybridization of a unique DNA probe to a complementary target sequence, target sequence amplification by PCR, microarray analysis to detect multiple sequences in a single sample, and mass spectrometry to identify single-nucleotide polymorphisms (SNPs).

Hybridization Probes:

Hybridization is the formation of hydrogen bonds between two complementary strands of nucleic acids. A diagnostic test involving DNA hybridization utilizes a DNA probe to detect a complementary target DNA sequence that is characteristic of the disease. The probe is labeled with a reporter molecule that indicates hybridization between the target and probe DNA. Hybridization probes are often employed to detect the presence of microbial pathogens. Malaria, caused by the parasite *Plasmodium falciparum*, is one of the most common infectious diseases and is especially fatal in young children.

A DNA diagnostic test for active infections that measures the presence of the pathogen was developed by using highly repetitive DNA sequences (present in many copies) from *P. falciparum*. The DNA sequence that was chosen as a specific probe hybridized with *P. falciparum* but not with *Plasmodium vivax*, *Plasmodium cynomolgi*, or human DNA, despite the fact that *P. vivax* causes a less severe form of malaria. This probe can detect as little as 10 picograms (pg) of purified *P. falciparum* DNA or 1 nanogram (ng) of *P. falciparum* DNA in blood.

45-Allele specific Hybridization

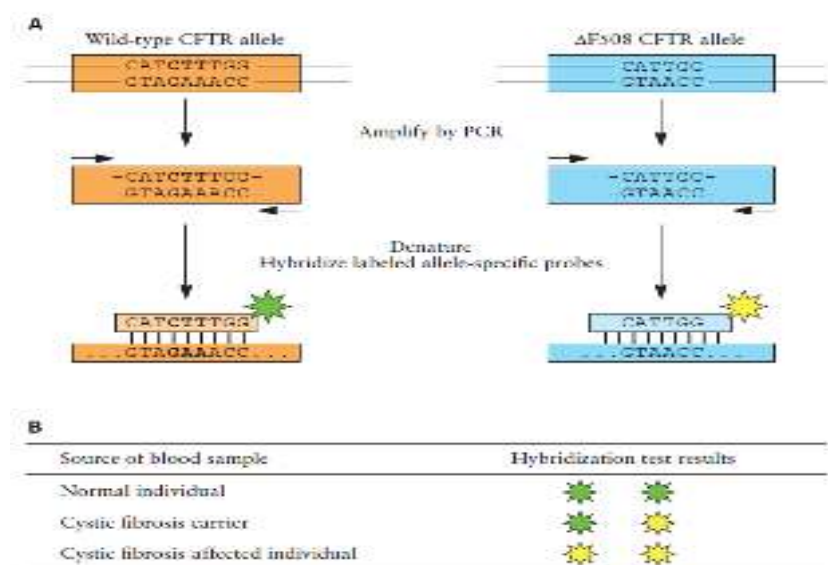
In addition to infectious diseases, hybridization probes are widely used to detect specific disease-associated alleles. Monogenic diseases are caused by mutations in a single gene; however, any one of several alterations to the normal nucleotide sequence of a gene may be responsible.

This is exemplified by cystic fibrosis, a common lethal autosomal recessive disorder that affects approximately 1 in every 2,500 live births. Mutations in a single gene, the cystic fibrosis transmembrane conductance regulator (CFTR) gene, result in defects in chloride ion transport.

As a consequence, the mucus of lung and other mucosal tissues is thick and viscous, obstructing the respiratory, digestive, and reproduction system functions. Every state in the United States now routinely screens newborns for cystic fibrosis. An ELISA is used to detect higher-than-normal levels of immunoreactive trypsinogen in blood. This protein is produced by the pancreas and is linked to cystic fibrosis. Premature babies and babies from stressful births also may have elevated levels of immunoreactive trypsinogen, and in these cases, genetic tests are employed for confirmation.

About 1,900 different mutations are reported to occur in the CFTR genes of patients with cystic fibrosis. Screening individuals who may be at risk for cystic fibrosis for such a large number of possible mutations is a daunting task. However, some of the mutations that cause cystic fibrosis are much more common than others. The most common mutation is an in-frame deletion of three nucleotides in exon 10 of the CFTR gene that leads to loss of the amino acid phenylalanine at codon 508 ($\Delta F508$). Over 90% of cystic fibrosis patients carry at least one $\Delta F508$ allele, and nearly 50% of cystic fibrosis patients are individuals who are homozygous for $\Delta F508$. It is estimated that about 160 different mutations account for 96 to 97% of cystic fibrosis alleles.

Allele-specific hybridization is commonly used to screen for cystic fibrosis. With this technique, an individual's CFTR gene is amplified by PCR and then hybridized to labeled oligonucleotide probes for the mutant (e.g., $\Delta F508$) and wild-type genes, separately (Fig. A). In this way, it is possible to distinguish between healthy individuals with two wild-type alleles, cystic fibrosis carriers with one mutant and one wild-type allele, and cystic fibrosis-affected individuals with two mutant alleles (Fig. B).



- (A) The CFTR gene is amplified by PCR, and then the PCR products are denatured and incubated with oligonucleotide probes that specifically hybridize to wild-type or mutant (usually $\Delta F508$) alleles (note that only part of the sequence of the CFTR gene and probe are shown). After a washing step to remove unbound probe, hybridization is detected by measuring fluorescence emitted by the fluorophore attached to the probe.
- (B) Healthy individuals are homozygous for the wild-type allele, while those affected by the disease carry two mutant alleles. Cystic fibrosis carriers are heterozygous for the CFTR alleles.

Diagnostic kits are commercially available that test patient blood samples for the presence of a panel of common CFTR mutations as recommended by the American College of Medical Genetics.

46-Oligonucleotide ligation assay

The oligonucleotide ligation assay (OLA) is also commonly used to detect SNPs known to be associated with human diseases with a high degree of accuracy. In this diagnostic assay, two short oligonucleotide probes (~50 nucleotides) are designed to anneal to adjacent sequences within a gene that encompass the polymorphic nucleotide (Fig.).

Importantly, one of the probes (allele-specific probe) has as its last base at the 3' end the nucleotide that is complementary to the polymorphic nucleotide. The second probe (common probe) is complementary to the sequence immediately downstream of the polymorphic nucleotide. When these two probes are hybridized with the target gene (which has been amplified by PCR), base-pairing occurs between the allele-specific probe and the target sequence, including the 3' nucleotide if the complementary polymorphic nucleotide is present, and the common probe binds immediately downstream. DNA ligase, added to the reaction, covalently joins the two probes. However, if the nucleotide at the 3' end of the allele-specific probe is mismatched, it will not base-pair with the polymorphic nucleotide in the target DNA sequence, although the common probe will be perfectly aligned. As a consequence of the single-nucleotide misalignment, DNA ligase cannot join the two probes.

In short, OLA is designed to distinguish between two possibilities: ligation if the probes are perfectly matched and no ligation if the allele-specific probe carries a mismatched nucleotide. Other oligonucleotide probes may be designed to detect different SNPs in the same gene. To determine whether ligation has occurred, one of the two probes is labeled with a reporter molecule. For example, the allele-specific probe may be labeled at the 5' end with a fluorescent dye that can be detected by laser excitation and fluorescence emission. Probes may be labeled with different fluorescent molecules corresponding to different SNPs. If an individual is homozygous for a particular allele, either two copies of a normal gene or two copies of a disease-associated SNP, a positive signal will be detected only from one of the

fluorescent molecules. Heterozygous individuals will yield positive signals from two different probes. Overall, the OLA system is rapid, sensitive, highly specific, and amenable to automation.

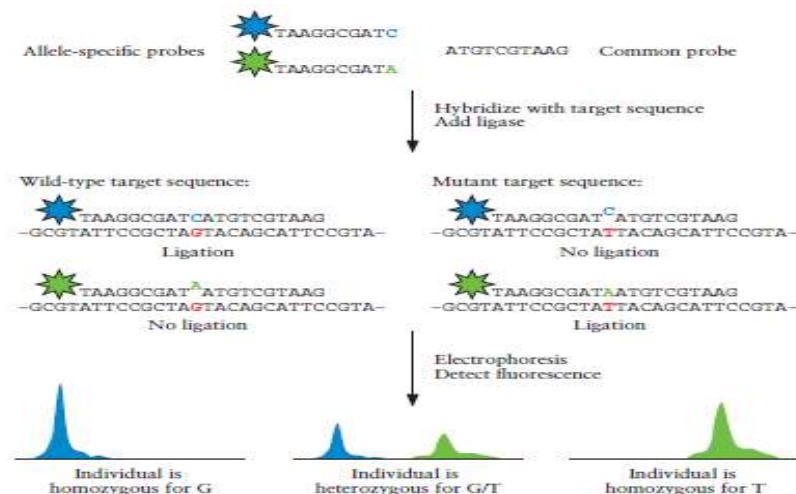


Fig-OLA. Ligation of the allele-specific and common probes occurs only when the allele-specific probe is perfectly complementary to the target sequence. Longer ligated probes may be separated from shorter unligated probes by electrophoresis, and then probe fluorescence can be detected to distinguish homozygous wild-type, homozygous mutant, and heterozygous individuals.

47-Padlock Probes

Diagnostic assays for SNPs that use padlock probes are very similar to those that use OLA probes, except that the former utilizes only one probe rather than two as used in the OLA procedure.

A padlock probe is an oligonucleotide that is complementary to a target sequence at its 5' and 3' ends but not in its middle region (Fig.). When a padlock probe hybridizes to its target sequence, the 5' and 3' ends of the probe come into close proximity to each other and the middle portion loops out. If the ends of the probe are exactly complementary to the target sequence, after hybridization they can be joined together by DNA ligase. If there is a mismatch between the target and probe, ligation does not occur.

The requirement for both ends of the probe to bind perfectly to the target sequence for ligation to occur ensures a high specificity of detection and therefore the ability to easily detect SNPs. Following the ligation reaction, the probe–target hybrid can be detected by the activity of reporter molecules attached to the middle (linker) portion of the probe. Padlock probes typically have sequences approximately 15 to 20 nucleotides in length at each end that are complementary to the target sequence and a middle region of approximately 50 nucleotides.

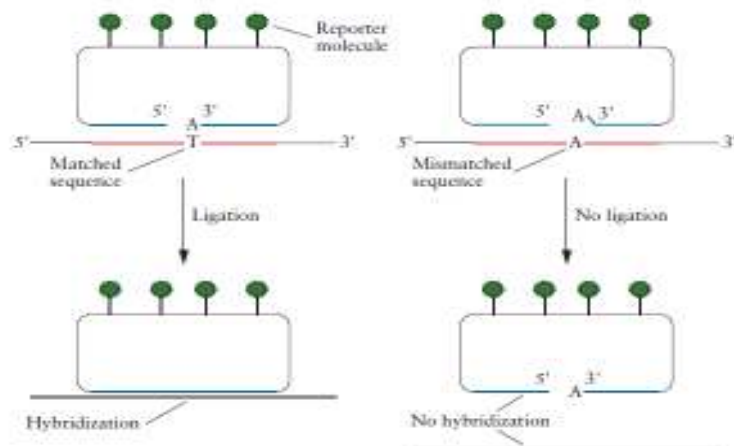


Fig-A padlock probe. Ligation (circularization) of the padlock probe occurs only when the 5' and 3' ends of the probe are perfectly complementary to the target sequence. When there is a single-base mismatch at the 3' end of the probe, ligation does not occur and the probe assumes a conformation that does not allow hybridization. Under stringent conditions, the ligated probe remains bound to the target DNA, while a nonligated probe is removed in a washing step. Hybridized probe can be detected by the activity of the reporter molecule, for example, by fluorescence emitted from a fluorescent dye.

48-Allele-Specific PCR

Many nucleic acid diagnostic tests are based on PCR. Advantages of PCR-based tests include:

- (i) specificity that enables detection of a particular nucleotide sequence in complex samples,
- (ii) sensitivity that enables detection of low-abundance targets,
- (iii) an amplification step that generates substantial amounts of a target sequence for additional analyses such as hybridization or sequencing,
- (iv) rapid analysis (usually completed in 1 to 2 h or less),
- (v) multiplexing that enables identification of multiple targets in a single sample, and
- (vi) low cost.

Conventional PCR has been used extensively in diagnostic laboratories over the last 15 years; however, more recently, it has become possible not only to detect but also to quantify the pathogen in a clinical sample using quantitative real-time PCR.

Allele-specific PCR (also referred to as PCR amplification refractory mutation system) screens for known SNPs. Different forward PCR primers are used to distinguish among alleles that differ by a single nucleotide. One primer is exactly complementary to the normal DNA sequence, and another primer anneals to a variant sequence containing the disease-associated SNP.

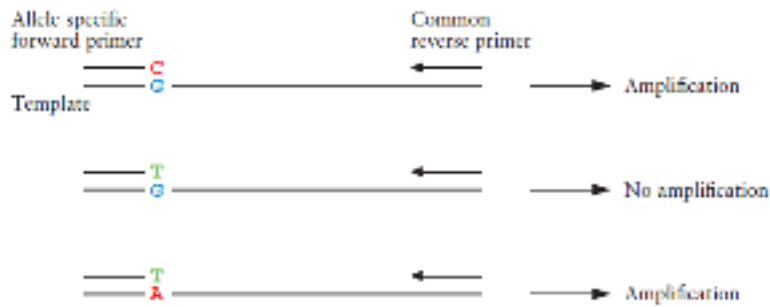


Fig- Allele-specific PCR to detect SNPs. Amplification occurs only when the forward and reverse PCR primers perfectly match the target sequence. Different allele-specific primers that carry an SNP-specific nucleotide at the 3' end are employed to distinguish among alleles that differ by a single nucleotide.

The primers are usually designed to place the polymorphic nucleotide at the 3'-terminal end of the primer because most polymerases used for PCR do not extend 3' mismatched primers efficiently. A third reverse primer is complementary to the opposite strand and is common to all reactions. Each reaction contains only one of the allele-specific forward primers and the common reverse primer, together with the patient DNA sample, a thermostable DNA polymerase, and all four deoxyribonucleotides.

PCR amplification occurs only when a forward primer is present that is exactly complementary to the target sequence in the patient sample; mismatches between primer and template DNA prevent primer annealing and therefore primer extension during DNA synthesis. An advantage of this method, compared to allele-specific hybridization described above, is that the amplification and diagnostic steps are combined.

One variation of allele-specific PCR is known as competitive oligopriming, in which two different SNP-specific forward primers are included in a single reaction. To discriminate between the PCR products, each forward primer is labeled with a different fluorescent dye. For example, one forward primer that is exactly complementary to a normal allele may be labeled at its 5' end with rhodamine (which fluoresces red), while another forward primer that is complementary to the disease-associated allele may be labeled at its 5' end with fluorescein (which fluoresces green)

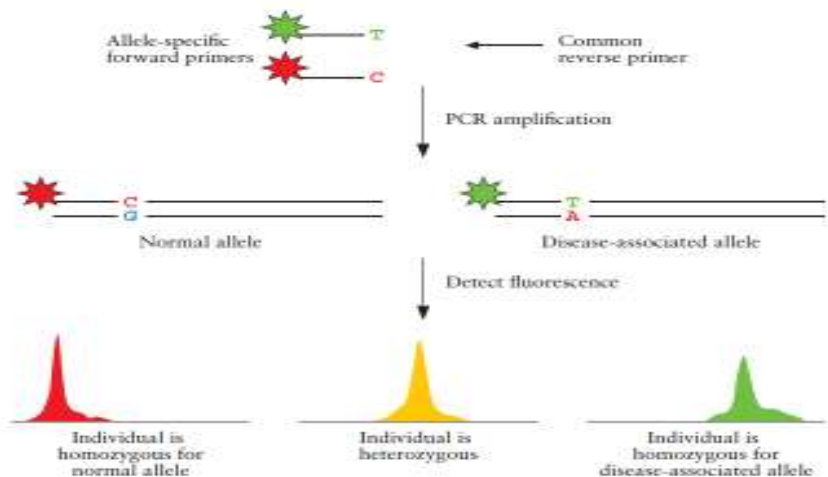


Fig- Competitive oligopriming to detect SNPs. Two allele-specific forward primers that differ in the nucleotide at the 3' end are included in a single reaction together with a reverse primer that anneals to a sequence that is common among variant alleles. PCR amplification occurs only when a forward primer is perfectly complementary to the target DNA. To discriminate between PCR products corresponding to normal and disease-associated alleles, each forward primer is labeled with a different fluorescent dye. In the example shown here, PCR products from individuals who are homozygous for the normal allele fluoresce red, those from individuals who are homozygous for the disease associated allele fluoresce green, and those from individuals who are heterozygous fluoresce yellow.

In both cases, amplification requires a third, unlabeled primer that is complementary to the opposite strand. PCR amplification occurs only when a forward primer is exactly complementary to the target DNA; therefore, the presence of these two forward primers in the same reaction mixture will result in the amplification of either the normal or disease-associated DNA sequence or both, depending on which alleles are present. If an individual is homozygous for the normal allele, after PCR and removal of unincorporated primers, the reaction mixture will fluoresce red; if he or she is homozygous for the disease-associated allele, the reaction mixture will fluoresce green; and if he or she is heterozygous, the reaction mixture will fluoresce yellow. This assay can be automated and adapted for any single-nucleotide target site of any gene that has been sequenced.

49-TaqMan PCR

The TaqMan PCR protocol is used to screen individuals for the presence of SNPs that are indicative of any of a variety of genetic diseases. Made popular by one particular company, it is based on the 5' nuclease activity of *Taq* polymerase, which is commonly used to amplify DNA in PCR applications.

To simultaneously detect normal and disease-associated alleles, two TaqMan probes are utilized. Each probe is exactly complementary to either the normal or the disease-associated DNA sequence, and each probe has a different fluorescent dye attached to its 5' end (Fig.). Intact probes, whether unbound or bound to template DNA, do not fluoresce because of the presence of a quencher molecule at the 3' end of the probe.

PCR primers anneal to sequences that flank the probe hybridization site, and as PCR amplification proceeds, the TaqMan probe is displaced by the growing DNA strand. The 5' nuclease activity of the *Taq* polymerase degrades the 5' end of the TaqMan probe, thereby releasing the fluorescent dye and removing it from the proximity of the quencher molecule. Thus, only TaqMan probes that were previously bound to target DNA are degraded and subsequently fluoresce. Any mismatched probes that are not complementary to the SNP in the region where the TaqMan probe binds will be displaced but not cleaved, so they will not fluoresce.

By monitoring the fluorescence at two different wavelengths (one for each TaqMan probe), it is possible to distinguish individuals that are homozygous for each SNP (either normal or

disease associated) and heterozygous (carrying one normal and one disease-associated allele). This technique may be used to detect more than two different SNPs at the same time as long as the fluorescent dyes attached to each SNP-specific probe have well-separated, nonoverlapping fluorescence maxima.

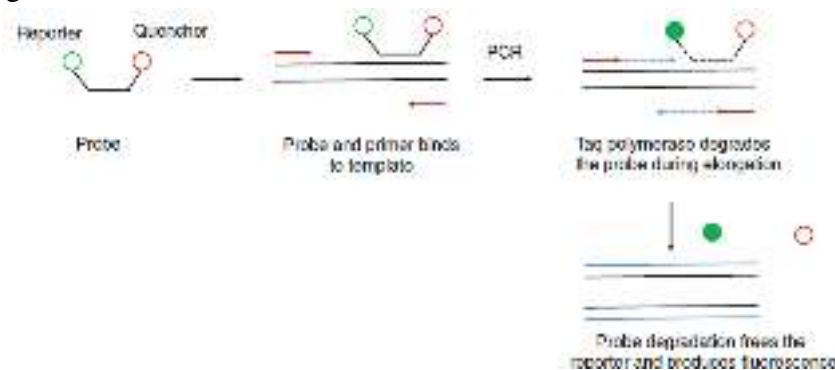
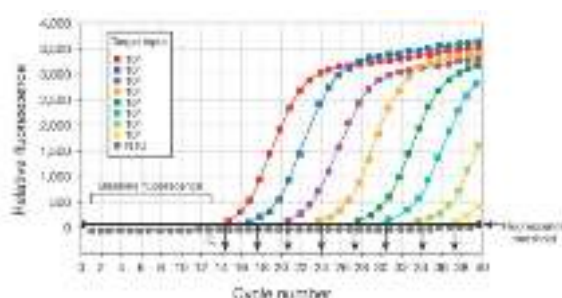


Fig-TaqMan q-PCR principle. A gene-specific probe with a reporter and quencher binds along with the primer. Since the reporter is close to the quencher, it does not produce fluorescence. During PCR reaction, Taq DNA polymerase degrades the probe due to 5' nuclease activity. The released reporter produces fluorescence signal that is captured after each cycle

50-Real-Time PCR To Detect Infectious Disease

Nucleic acid detection and accurate quantitation has become integral in all areas of scientific research. It has a wide range of applications in basic science, biotechnology, medicine, diagnostics, and forensic science. q-PCR is a valuable technique for detection and quantification of DNA and RNA. Unlike endpoint PCR where the amplicon is quantitated and detected at the end of the reaction, in real-time PCR, the amount of DNA is measured after each cycle based on increasing yield of fluorescent signal produced by the accumulation of fluorescent dye.

The signal is directly proportion to the number of molecules generated. Data collected in the exponential phase of the reaction yield quantitative information on the starting quantity of the target. The change in fluorescence is measured by a thermal cycler that has fluorescent dye scanning capability. By plotting fluorescence against the cycle number, it generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction.

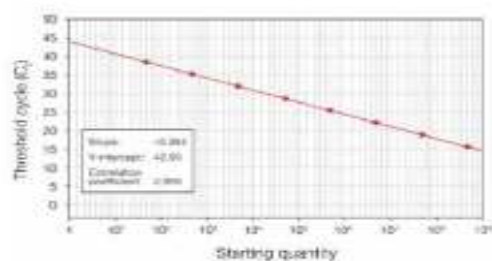


The steps in q-PCR are similar to that of conventional PCR . It includes denaturation of the DNA template, followed by primer annealing and extension. In case of RNA quantitation, a reverse transcription step is done before PCR where the RNA is reverse transcribed to complementary DNA (cDNA). The cDNA is used as a template for q-PCR. After the completion of every cycle, the fluorescence signal is measured and plotted against the cycle number to provide an amplification curve (Fig.). Amplification curve has two phases: exponential and non-exponential phase. In exponential phase, the PCR product doubles after every cycle. Hence the fluorescence signal increases exponentially. The cycle number at which the fluorescence signal reaches detectable limit is called as threshold cycle or C T . Since C T is calculated at exponential phase when the reagents are not limiting, it can be used to calculate the initial amount of template accurately and reliably.

C T value is inversely related to the starting quantity of the template. The more the amount of the template, the earlier it will reach the C T . q-PCR can be used for exact quantitation or relative quantitation of a target. For exact quantitation, it is important to create a standard curve with known amount of the target. This can be done by performing q-PCR reaction with serial dilution of the purified target. Log of known concentration of the target template is plotted against the corresponding C T value to prepare the standard curve. This is used for determining the exact quantity of the target template in the experimental sample and also to calculate the efficiency of the reaction. Most of the time, relative quantitation is done where the expression of the target template is calculated as fold changes with regard to an internal control that could either be a single- copy gene or a housekeeping gene that is expressed steadily in all tissue in every condition.

Standard curve

A dilution series of known template concentrations can be used to establish a standard curve for determining the initial starting amount of the target template in experimental samples or for assessing the reaction efficiency (Fig.) The log of each known concentration in the dilution series (x-axis) is plotted against the Ct value for that concentration (y-axis). From this standard curve, information about the performance of the reaction as well as various reaction parameters (including slope, y-intercept, and correlation coefficient) can be derived.



Correlation coefficient (R²)

The correlation coefficient is a measure of how well the data fit the standard curve. The R² value reflects the linearity of the standard curve. Ideally, R² = 1, although 0.999 is generally the maximum value.

Y-intercept

The y-intercept corresponds to the theoretical limit of detection of the reaction, or the Ct value expected if the lowest copy number of target molecules denoted on the x-axis gave rise to statistically significant amplification. Though PCR is theoretically capable of detecting a single copy of a target, a copy number of 2–10 is commonly specified as the lowest target level that can be reliably quantified in real-time PCR applications. This limits the usefulness of the y-intercept value as a direct measure of sensitivity. However, the y-intercept value may be useful for comparing different amplification systems and targets.

Slope: The slope of the log-linear phase of the amplification reaction is a measure of reaction efficiency. To obtain accurate and reproducible results, reactions should have an efficiency as close to 100% as possible, equivalent to a slope of -3.32

There are two types of PCR chemistry that is widely used for q-PCR:

- SYBR ® Green dye-based assay
- 5' nuclease assay or TaqMan ® Assay.

Most nucleic acid diagnostic tests for etiological agents of infectious disease are based on PCR. As for other molecular approaches, PCR circumvents the requirement to grow the pathogens, which is time-consuming for viruses and slow-growing or fastidious bacteria. Not only does this technique enable identification of a viral, bacterial, or fungal pathogen, but also it can reveal specific characteristics of that pathogen, for example, the presence of antibiotic resistance genes that can be used to determine the best course of treatment.

In a real-time PCR, the double-stranded DNA product is bound by a fluorescent dye and the fluorescence is measured after each amplification cycle to quantify the amount of product in real time. Post-PCR processing is not required, and therefore, time to pathogen identification is shorter than for conventional PCR.

51-DNA Sequencing Technologies

Determination of the nucleotide composition and order in a gene or genome is a foundational technology in modern medical biotechnology. DNA sequencing enables determination of the exact nucleotide sequence of a DNA fragment. DNA sequences can often reveal something about the function of the protein or RNA molecule encoded in a gene, for example, from predicted cofactor binding sites, transmembrane domains, receptor recognition sites, or DNA-binding regions. The nucleotide sequences in noncoding regions that do not encode a protein or RNA molecule may provide information about the regulation of a gene. Comparison of gene sequences among individuals can reveal mutations that contribute to genetic diseases or the relatedness among the individuals. Comparison of gene sequences among different organisms can lead to the development of hypotheses about the evolutionary relationships among organisms.

This technique was developed independently by two groups. Maxam and Gilbert invented DNA sequencing by chemical cleavage method, and Sanger developed dideoxy chain termination method for sequencing. Many different sequencing technologies have been developed, including **pyrosequencing**, sequencing using **reversible chain terminators**, and sequencing by ligation.

In general, all of these methods involve:

- (i) enzymatic addition of nucleotides to a primer based on complementarity to a template DNA fragment and
- (ii) detection and identification of the nucleotide(s) added. The techniques differ in the method by which the nucleotides are extended, employing either DNA polymerase to catalyze the addition of single nucleotides (sequencing by synthesis) or ligase to add a short, complementary oligonucleotide (sequencing by ligation), and in the method by which the addition is detected.

52-Dideoxynucleotide Procedure

The dideoxynucleotide procedure for DNA sequencing is based on the principle that during DNA synthesis, addition of a nucleotide triphosphate requires a free hydroxyl group on the 3' carbon of the sugar of the last nucleotide of the growing DNA strand (Fig. A).

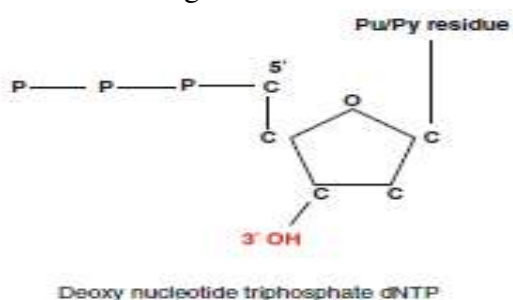


Fig-(A) Addition of an incoming deoxyribonucleoside triphosphate (dNTP) requires a hydroxyl group on the 3' carbon of the last nucleotide of a growing DNA strand.

However, if a synthetic dideoxynucleotide that lacks a hydroxyl group at the 3' carbon of the sugar moiety is incorporated at the end of the growing chain, DNA synthesis stops because a phosphodiester bond cannot be formed with the next incoming nucleotide (Fig. B).

The termination of DNA synthesis is the defining feature of the dideoxynucleotide DNA sequencing method.

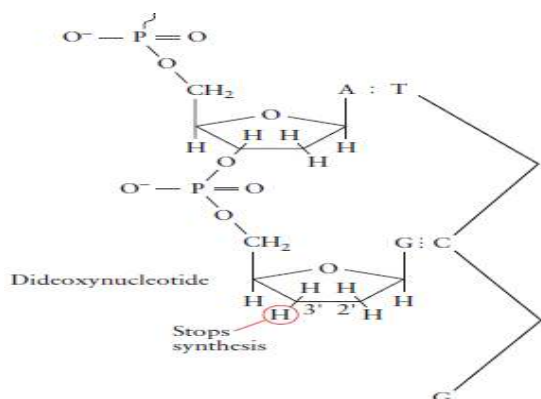


Fig- (B) DNA synthesis stops if a synthetic dideoxynucleotide that lacks a 3' hydroxyl group is incorporated at the end of the growing chain because a phosphodiester bond cannot be formed with the next incoming nucleotide.

In a dideoxynucleotide DNA sequencing procedure, a synthetic oligonucleotide primer (~17 to 24 nucleotides) anneals to a predetermined site on the strand of the DNA to be sequenced (Fig.A). The oligonucleotide primer defines the beginning of the region to be sequenced and provides a 3' hydroxyl group for the initiation of DNA synthesis. The reaction tube contains a mixture of the four deoxyribonucleotides (deoxyadenosine triphosphate [dATP], deoxycytidine triphosphate [dCTP], deoxyguanosine triphosphate [dGTP], and deoxythymidine triphosphate [dTTP]) and four dideoxynucleotides (dideoxyadenosine triphosphate [ddATP], ddCTP, ddGTP, and ddTTP). Each dideoxynucleotide is labeled with a different fluorescent dye. The concentration of the dideoxynucleotides is optimized to ensure that during DNA synthesis a modified DNA polymerase incorporates a dideoxynucleotide into the mixture of growing DNA strands at every possible position. Thus, the products of the reaction are DNA molecules of all possible lengths, each of which includes the primer sequence at its 5' end and a fluorescently labeled dideoxynucleotide at the 3' terminus (Fig. B).

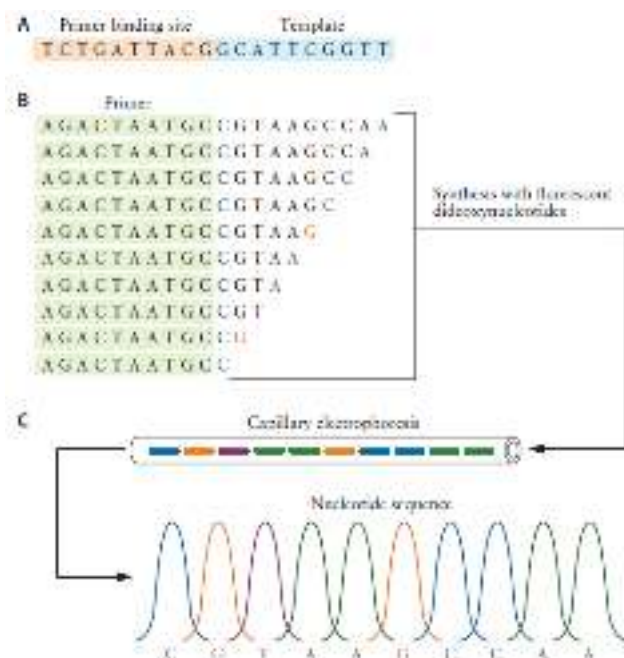


Fig- Dideoxynucleotide method for DNA sequencing. An oligonucleotide primer binds to a complementary sequence adjacent to the region to be sequenced in a single-stranded DNA template (A). As DNA synthesis proceeds from the primer, dideoxynucleotides are randomly added to the growing DNA strands, thereby terminating strand extension. This results in DNA molecules of all possible lengths that have a fluorescently labeled dideoxynucleotide at the 3' end (B). DNA molecules of different sizes are separated by capillary electrophoresis, and as each molecule passes by a laser, a fluorescent signal that corresponds with one of the four dideoxynucleotides is recorded. The successive fluorescent signals are represented as a sequencing chromatogram (colored peaks) (C).

PCR-based cycle sequencing is performed to minimize the amount of template DNA required for sequencing. Multiple cycles of denaturation, primer annealing, and primer extension produce large amounts of dideoxynucleotide-terminated fragments. These are applied to a polymer in a long capillary tube that enables separation of DNA fragments that differ in size

by a single nucleotide. As each successive fluorescently labeled fragment moves through the polymeric matrix in an electric field and passes by a laser, the fluorescent dye is excited. Each of the four different fluorescent dyes emits a characteristic wavelength of light that represents a particular nucleotide, and the order of the fluorescent signals corresponds to the sequence of nucleotides (Fig. C). The entire dideoxynucleotide sequencing process has been automated to increase the rate of acquisition of DNA sequence data. This is essential for large-scale sequencing projects such as those involving whole prokaryotic or eukaryotic genomes. Generally, automated DNA cycle sequencing systems can read with high accuracy about 500 to 600 bases per run.

LECTURE 53

54-Sequencing Using Reversible Chain Terminators

For pyrosequencing, each of the four nucleotides must be added sequentially in separate cycles. The sequence of a DNA fragment could be determined more rapidly if all the nucleotides were added together in each cycle. However, the reaction must be controlled to ensure that only a single nucleotide is incorporated during each cycle, and it must be possible to distinguish each of the four nucleotides. Synthetic nucleotides known as reversible chain terminators have been designed to meet these criteria and form the basis of some of the next-generation sequencing-by-synthesis technologies.

Reversible chain terminators are deoxynucleoside triphosphates with two important modifications: (i) a chemical blocking group is added to the 3' carbon of the sugar moiety to prevent addition of more than one nucleotide during each round of sequencing, and (ii) a different fluorescent dye is added to each of the four nucleotides to enable identification of the incorporated nucleotide (Fig. A).

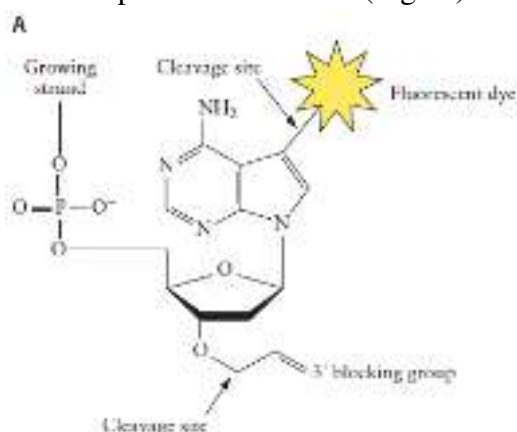


Fig-Sequencing using reversible chain terminators.

(A) Reversible chain terminators are modified nucleotides that have a removable blocking group on the oxygen of the 3' position of the deoxyribose sugar to prevent addition of more than one nucleotide per sequencing cycle. To enable identification, a different fluorescent dye is attached to each of the four nucleotides via a cleavable linker. Shown is the fluorescent dye attached to adenine.

The **fluorophore** is added at a position that does not interfere with either base-pairing or phosphodiester bond formation. Similar to the case with other sequencing-by-synthesis methods, DNA polymerase is employed to catalyze the addition of the modified nucleotides to an oligonucleotide primer as specified by the DNA template sequence (Fig. B).

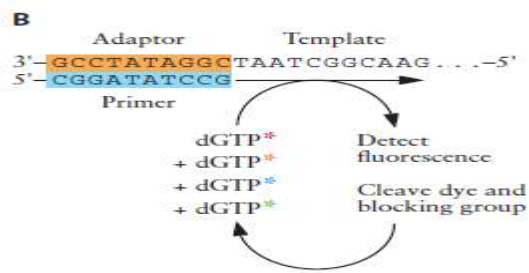


Fig-(B) An adaptor sequence is added to the 3' end of the DNA sequencing template that provides a binding site for a sequencing primer. All four modified nucleotides are added in a single cycle, and a modified DNA polymerase extends the growing DNA chain by one nucleotide per cycle. Fluorescence is detected, and then the dye and the 3' blocking group are cleaved before the next cycle. Removal of the blocking group restores the 3' hydroxyl group for addition of the next nucleotide.

After recording fluorescent emissions, the fluorescent dye and the 3' blocking group are removed. The blocking group is removed in a manner that restores the 3' hydroxyl group of the sugar to enable subsequent addition of another nucleotide in the next cycle. Cycles of nucleotide addition to the growing DNA strand by DNA polymerase, acquisition of **fluorescence** data, and chemical cleavage of the blocking and dye groups are repeated to generate short read lengths, i.e., 50 to 100 nucleotides per run.

55-Sequencing by Ligation

Pyrosequencing and sequencing using reversible terminators extend the growing DNA strand by a single base during each cycle. In contrast, sequencing by ligation extends the DNA strand by ligation of short oligonucleotides, in a template-dependent fashion, and utilizes the enzyme ligase rather than DNA polymerase. In one version of this technology, the oligonucleotides are eight nucleotides in length (octamers) with two known nucleotides at the 3' (query) end, any nucleotide in the next three (degenerate) positions, and a sequence that is common (universal) to all of the oligonucleotides at the 5' end (Fig.A). A set of 16 different oligonucleotides representing all possible combinations of two nucleotides in the query position is used. Each oligonucleotide is tagged at the 5' end with a different fluorescent dye that corresponds to the query nucleotide composition.



Fig-(A) Pools of octamers. Each octamer has two known nucleotides at the 3' query end (colored letters), any nucleotide in the next three positions (NNN), and a universal sequence that is common to all of the octamers at the 5' end (ZZZ). A set of 16 different octamers representing all possible combinations of two nucleotides in the query positions is shown.

Each of the four octamers in a pool (each pool is shown in column) is tagged at the 5' end with a different fluorescent dye that corresponds to the query nucleotide composition.

A short nucleotide adaptor is joined to the 5' ends of the DNA templates that are to be sequenced (Fig. B). After denaturation of the template DNA, a primer binds to the adaptor sequence and provides a 5' phosphate end for ligation to the 3' hydroxyl end of an adjacent hybridized octamer. Sequential cycles of octamer ligation extend the complementary strand, and each cycle enables determination of the query dinucleotide sequence (Fig. B).

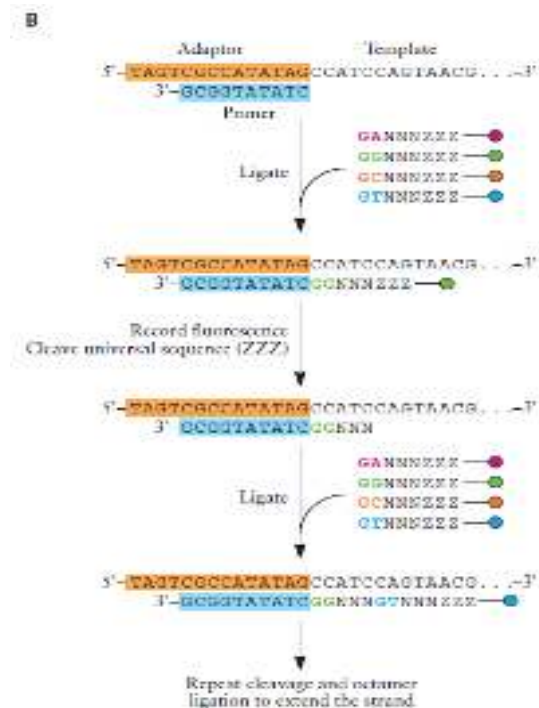


Fig-(B) To determine a sequence by ligation, an adaptor is first added to the 5' end of the template DNA molecule. The adaptor provides a binding site for a sequencing primer. Pools of octamers are added, and an octamer that contains a nucleotide sequence that is complementary to the first five bases immediately adjacent to the primer will hybridize. T4 DNA ligase catalyzes the formation a phosphodiester bond between the 5'phosphate group of the primer and the 3' hydroxyl group of the hybridized octamer. After unbound octamers are washed away, the fluorescent signal from the ligated octamer is recorded to identify the dinucleotides in the query position. The fluorescent dye is removed by cleavage of the universal sequence (ZZZ) to expose a 5' phosphate for ligation of another octamer. The cycle of octamer ligation and dye removal is repeated to extend the DNA strand and identify discontinuous dinucleotides that are separated by three nucleotides (NNN).

One ligation cycle consists of the following steps.

- Pools of different octamers, each tagged with a different fluorescent dye, are added along with T4 DNA ligase. Reaction conditions are set so that ligation occurs only if the bases at the 3' end of an octamer (i.e., the query and degenerate nucleotides) are complementary to the template sequence.
- After washing away of the nonligated octamers and other components, the fluorescence signal is recorded. The identity of the dinucleotides in the query position is determined by the distinctive fluorescence emitted.
- The fluorescent dye is removed after each cycle by cleaving the terminal universal nucleotides. Cleavage provides a free end for ligation of another octamer in the next ligation cycle.

Successive cycles of ligation extend the complementary strand and enable identification of discontinuous nucleotide pairs that are separated by three nucleotides (Fig. B). The length of the sequence read is determined by the number of ligation cycles (~50 bp). To determine the nucleotide sequence of the intervening regions (i.e., between the identified dinucleotides), the primer and octamers are removed from the template strand, and the process is repeated using

a new primer that is set back on the template DNA by one nucleotide from the first primer (Fig. C). In total, the entire process of primer extension by serial octamer ligation is repeated five times, each time using a different primer that is offset from the previous primer by one nucleotide (Fig. C). In this way, the nucleotide in each position in the template DNA is identified twice in separate reactions, which increases the accuracy of the sequence determination.

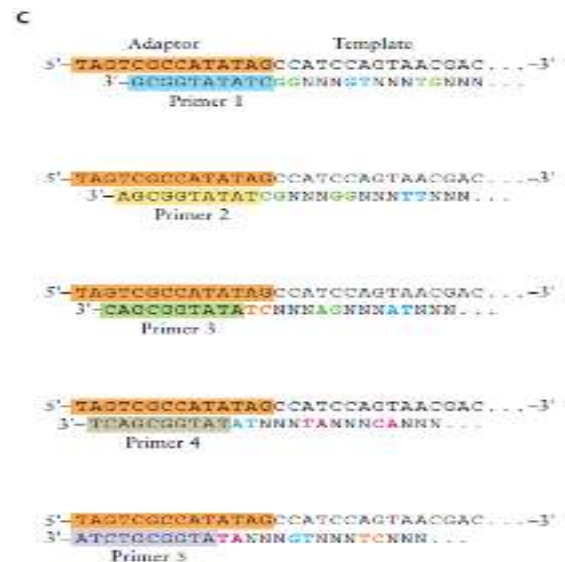


Fig-(C) To determine the identity of the intervening nucleotides, the primer and ligated octamers are removed and the entire process is repeated using a different primer that binds to a position on the adaptor onenucleotide from the previous primer-binding site. The process is repeated with five different primers. In this manner, each nucleotide in the template sequence is identified twice, once in two separate reactions.

56-The Immune Response

Overview of Infection and Immunity

Immunology is the study of the immune system and its responses to a host of invading pathogens and other harmful agents (e.g., insect toxins). The **immune system** is comprised of several types of cells, tissues, and molecules that mediate resistance to infection and accompanying infectious disease, and the interaction of these cells, tissues, and molecules to infectious microbes elicits an **immune response**. Thus, the raison d'être of the immune system is to initially prevent infection and then to arrest and eliminate established infection.

The immune system is essential to maintain the health of an individual, as demonstrated by the increased susceptibility of individuals with deficient immune responses to acute, invariably life-threatening infections. In contrast, the stimulation of immune responses against microbes by vaccination is the most effective method of protection of individuals against infection.

Table-1 Role of the immune system in health and disease

Defensive role	Consequence
Infection	Reduced immunity increases susceptibility to infection (e.g., HIV). Vaccination increases immunity and protects against infections.
Immune responses to tissue transplants and novel proteins	Barriers to transplantation and gene therapy
Tumors	Possible immunotherapy for cancer

Table-2 Benefits of vaccination for some infectious diseases

Disease	Highest no. of cases (yr)	No. of cases in 2004	Decrease (%)
Diphtheria	206,939 (1921)	0	100.0
<i>Haemophilus influenzae</i> type b infection	20,000 (1984)	16	99.9
Hepatitis B	26,611 (1985)	6,632	75.1
Measles	894,134 (1941)	37	99.9
Mumps	152,209 (1968)	236	99.9
Pertussis	265,269 (1934)	18,957	96.8
Polio (paralytic)	21,269 (1952)	0	100.0
Rubella	57,686 (1969)	12	99.9
Tetanus	1,560 (1923)	26	98.3
TB	17,500 (1993)	14,511	96.7

Notably, by 1977, smallpox, long considered to be the most deadly and persistent human pathogenic disease, was eradicated by the World Health Organization (WHO). This was accomplished through a massive, worldwide outbreak search and vaccination program. The underlying benefit of such immunizations is that the vaccine triggers an immune response more rapidly than the natural infection itself. The emergence of acquired immunodeficiency syndrome (AIDS) since the 1980s has further emphasized the importance of the immune system for defending individuals against infection.

Importantly, the impact of immunology extends beyond infectious disease. For example, the immune response is the major barrier to successful organ transplantation, which is being used more frequently as a therapy for organ failure. Attempts to treat cancers by stimulating immune responses against cancer cells are being tried for many human malignancies. Furthermore, abnormal immune responses are the causes of many inflammatory diseases with serious morbidity and mortality. Antibodies, protein products synthesized by B cells during an immune response, are highly specific reagents for detecting a wide variety of molecules in the circulation and in cells and tissues. Therefore, antibodies have emerged as valuable reagents for the development of laboratory diagnostic technologies for both clinical research and clinical trials. Antibodies designed to block or eliminate potentially harmful molecules and cells are in extensive use for the treatment of immunological diseases, cancers, and other types of disorders. Accordingly, the field of immunology impacts highly on the interests of clinicians, scientists, and the lay public.

57-Functions of the Immune System

Four important functions are performed by the immune system (Table 1).

1. First, **immunological recognition** is conducted by **leukocytes** (e.g., neutrophils, macrophages, and **natural killer cells**) of the **innate immune system** that provide an early rapid response and by the **lymphocytes** (T cells and B cells) of the later, more antigen-specific and more efficient **adaptive immune system**.
2. Second, to contain and eliminate the infection, **immune effector functions** that involve the complement system of blood proteins, enzymes, and antibodies together with certain T cells and B cells are required.
3. Third, while combating foreign pathogens, the immune system must learn not to elicit damage to self-tissues and other components of the body. It accomplishes this by **immune regulation**, a process in which regulatory lymphocytes control various immune responses against self-components. A failure in immune regulation could potentially result in allergy and autoimmune disease.
4. Fourth, the immune system must be equipped to protect an individual against recurring disease upon exposure to the same or a closely related pathogen. This task is uniquely mediated by the adaptive immune system during a response known as **immunological memory**. After exposure to an infectious pathogen, a person will make a rapid and more vigorous response to the pathogen upon subsequent encounter with this pathogen and will acquire protective immunity against it. Thus, a very significant challenge facing immunologists today is the identification of relevant cellular pathways and molecule that generate long-lasting immunity (i.e., memory) to pathogens, with the aim of identifying novel **immunotherapies** using cells and/or antibodies that eradicate such pathogens.

Table-1 Functions of the immune system

Function	Cell type
Immune recognition	Leukocytes (neutrophils, macrophages, and natural killer cells) and lymphocytes (T cells and B cells)
Contain and eliminate infection	Effector cells (T cells and B cells) and molecules (complement proteins, enzymes, and antibodies)
Immune regulation	Regulatory lymphocytes
Immunological memory	Memory lymphocytes

Two major types of immunity are carried out by cells of the immune system in response to inflammation and infection (Fig.1). First, **innate immunity** provides a mechanism of host defense that mediates the initial protection against microbial infections. Second, **adaptive immunity**, which develops more slowly and confers specificity against a foreign antigen, mediates the later and more vigorous defense against infections.

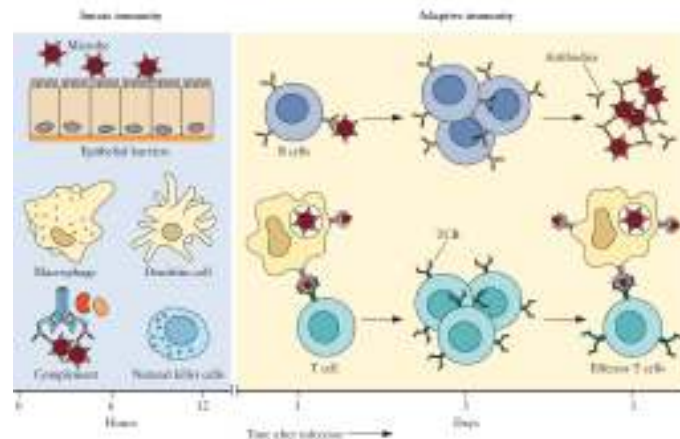
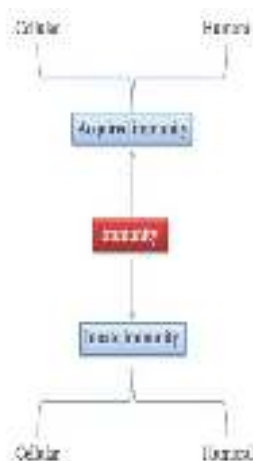


Fig-1 Mechanisms of innate and adaptive immunity. The initial defense against infections is mediated by mechanisms of innate immunity that either protect from infection (mucosal epithelial barriers) or eliminate microbes (macrophages, natural killer [NK] cells, and complement system). Adaptive immune responses develop later and are mediated by lymphocytes and their products. While antibodies block infections and eliminate microbes, T cells eradicate intracellular microbes. The kinetics of innate (early after infection) and adaptive (late after infection) immune responses are different and may vary with the type of infection incurred.

58. Innate immune system

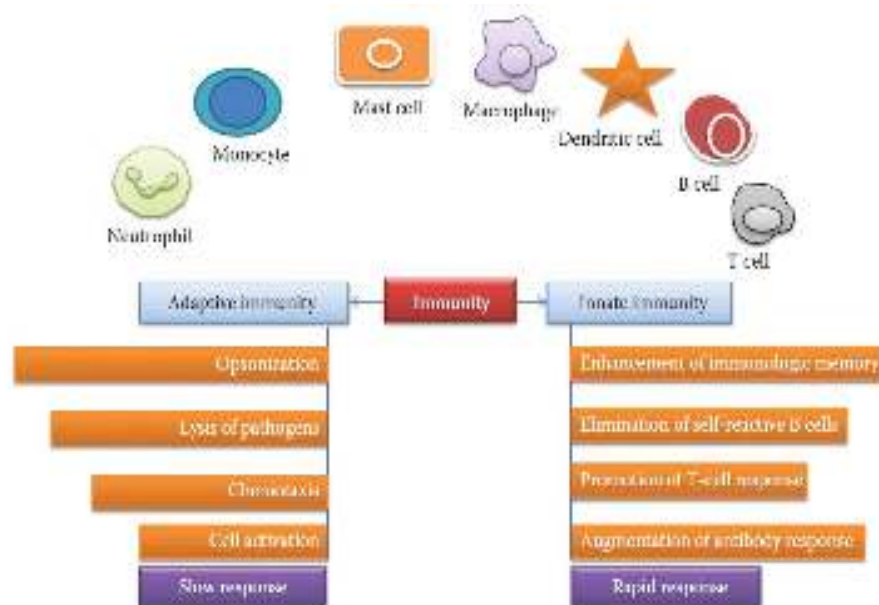
The innate immune system contains cells and mechanisms that defend or fight against infection by other microorganisms in a generalized manner, which suggests that the cells of the innate system identify and respond to pathogens in a common way, and it does not convene long-lasting or protective immunity to the host



It has been reported that innate immune systems offer immediate defense against infection and are found in all plants and animals. It has been found that the innate immune system generally consists of three components—membrane barrier, inflammation, and complement system—to protect the cells from infections

In case pathogens can successfully evade the innate response, a host body possesses another layer of protection called the adaptive immune system, which can be activated by the innate response. At this point, the immune system familiarizes its response during an infection to increase its recognition of the pathogen.

Difference between innate and adaptive (acquired) immunity



Types of immune diseases



59-Cellular network of innate immune system

The white blood cells (WBCs), identified as leukocytes, are different from other cells of the body because they are normally associated with a particular organ. It has been reported that leukocytes are able to move freely in the body and network with cellular debris, foreign particles, and invading pathogenic microorganisms. Interestingly, most innate leukocytes cannot multiply or reproduce on their own, unlike other body cells. These leukocytes are the products of hematopoietic stem cells present in the bone marrow and we briefly describe the various types of innate leukocytes.

Mast cells

In the human body, mast cells are basically innate immune cells that reside in the connective tissue and in the mucous membranes, and these cells are closely associated with defense against pathogens and wound healing. After activation, mast cells speedily release some granules that are equipped with histamine and heparin and chemotactic cytokines into the host cell environment. It has been reported that histamines open blood vessels, causing irritation or inflammation, and recruit neutrophils and macrophages to defend the host cells (Figure 1).

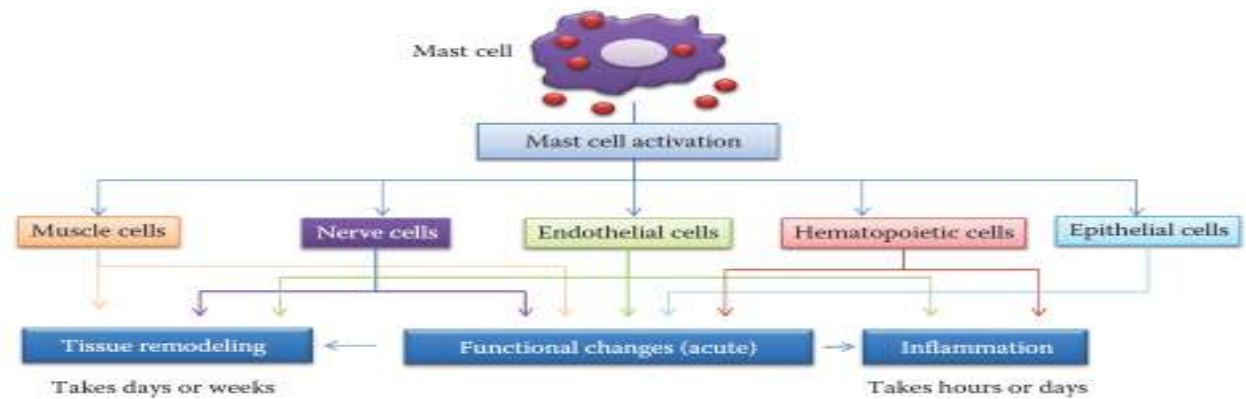


Fig-1 Mast cells and their role in innate and acquired immunity.

Phagocytes: Phagocytes are basically immune cells that engulf pathogens or particles to protect the body from any infection. It has been reported that phagocytes engulf a particle or pathogen by extending parts of its plasma membrane and wrapping the membrane around the particle until it is completely enveloped and covered. Once the pathogen is completely covered, the lysosome, which contains enzymes and acids, starts killing and digesting the foreign particle or organism. It has been described that phagocytes generally patrol the human body searching for pathogens, and are also able to respond to molecular signals produced by other cells, which are called cytokines. There are various types of phagocytic cells reported in the human immune system that include neutrophils, macrophages, and dendritic cells (Figure 2).

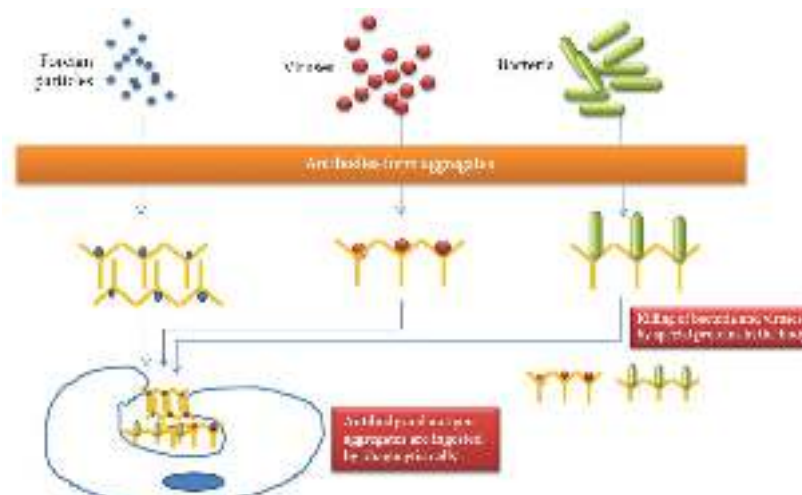


Fig-2 Role of phagocytic cells in immune protection.

60-Macrophages

Macrophages are large leukocytes that are able to move outside the vascular system and enter the areas between cells in the hunt of invading pathogens. It has been reported that these macrophages are the most efficient phagocytes, and can kill large numbers of bacteria or other cells or microorganisms. The attachment of anti-bacterial molecules to receptors of a macrophage terminates the bacterial population through respiratory spurt, causing the release

of reactive oxygen species. It has been reported that pathogens can also stimulate the macrophage to produce chemokines, which command other cells to the site of infection.

Neutrophils

Because of the presence of granules in their cytoplasm, neutrophils, eosinophils, and basophils are known as granulocytes. Neutrophils are also known as polymorphonuclear cells (PMNs) due to their typical lobed nuclei. It has been reported that neutrophil granules contain a variety of toxic elements that destroy or inhibit the growth of bacteria and fungi. Like macrophages, neutrophils attack pathogens by activating a respiratory spurt that contains oxidizing agents, including free oxygen radicals, hydrogen peroxide, and hypochlorite. It has been reported that neutrophils are the most plentiful type of phagocyte, normally representing 50–60% of the total mobile leukocytes, and they are usually the first type of cells to reach the site of an infection. It has been estimated that the bone marrow of a healthy adult human being produces more than 100 billion neutrophils per day, and produces more than 10 times more neutrophils during acute inflammation or infection.

Dendritic cells

Dendritic cells are phagocytic cells present in human tissues that are in contact with the external atmosphere, primarily the skin, where they are referred to as Langerhans cells, and the inner mucosal lining of the nose, stomach, intestines, and lungs. The structure of dendritic cells closely resembles neuronal dendrites; that is why they are called dendritic cells. They have no association with neuronal cells. It has been suggested that dendritic cells are very vital in antigen performance and assist as a link between the innate and adaptive immune defense system (Figure 3).

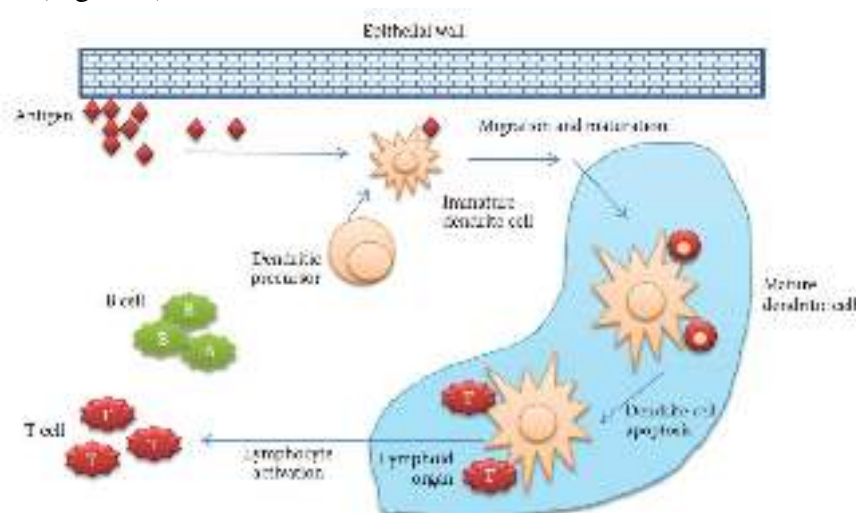


Fig-3 Dendritic cells and their biological actions.

Basophils and eosinophils: Basophils and eosinophils are known to relate to the neutrophils. Moreover, the basophils release histamine upon parasite entry, which are critical in the defense against parasites, and are also involved in allergic reactions. These basophils during activation secrete a highly toxic protein and free radicals that instantly kill bacteria and

parasites; however, they are also known to cause cell and tissue damage during allergic-related reactions.

Natural killer cells: Natural killer (NK) cells are an important member of the innate immune defense system that does not attack invading pathogens or microbes. In contrast to phagocytes, NK cells can terminate compromised host cells, such as cancerous cells or virus-infected cells. They are called NK cells because of an initial idea that these NK cells do not require activation to kill cells (Figures 4).

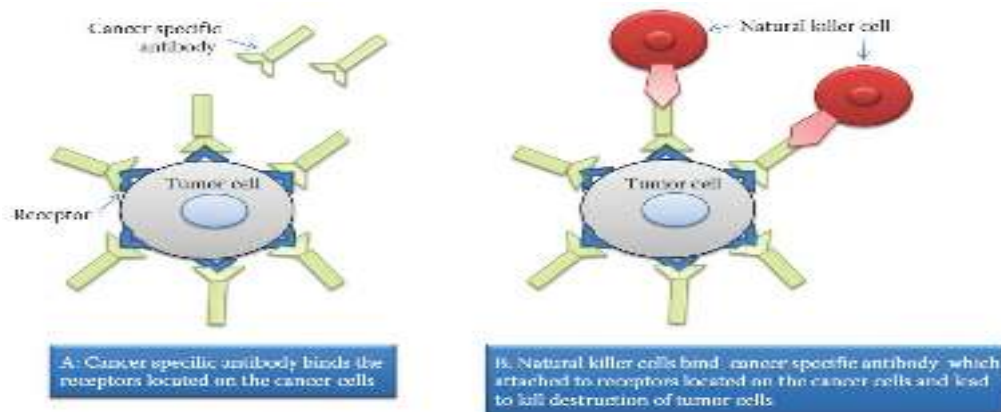


Fig 4- NK cell destroying cancerous cells.

61-Adaptive immune system

The adaptive immune system is basically a defense system in the human body that remembers the entry and action of each and every pathogen. Moreover, adaptive immune response is antigen specific and needs the recognition of specific antigens during antigen presentation. Antigen specificity can allow the generation of responses that are tailored to specific pathogens or pathogen-infected cells in the body. The special adaptive capability of immune cells is maintained in the body by memory cells.

Lymphocytes: The lymphocytes belong to the adaptive immune system. There are two different types of lymphocytes, B cells and T cells. These cells are generally derived from hematopoietic stem cells in the bone marrow. Moreover, B cells take part in the humoral immune response, while T cells are mainly involved in cell-mediated immune response. Both B and T cells contain receptor molecules that distinguish specific targets. For example, T cells recognize a pathogen only after antigens are processed and presented in combination with major histocompatibility complex (MHC) molecule. There are two major subtypes of T cells—killer T cells and helper T cells. Killer T cells can only identify antigens coupled to Class-I MHC molecules, while helper T cells only identify antigens coupled to Class-II MHC molecules. A third, minor subtype is the gamma–delta T cells that distinguish integral host antigens that are not attached to MHC receptors.

62-Killer T cells

Killer T cells are a subtype of T cells that can either kill cells that are infected with viruses and pathogens, or make them dysfunctional. It has been shown that killer T cells are activated

when T cell receptor binds to antigens of another MHC cell. Moreover, the recognition of this MHC–antigen complex is aided by a coreceptor localized on the T cell, which is also called CD-8. It has been suggested that T cells travel throughout the body in hunt of cells where the MHC-I receptors contain this antigen. As soon as an activated T cell makes contact with such cells, it releases cytotoxins that form holes in the target cell's plasma membrane, and subsequently allow ions, water, and toxins to enter inside the cell. Furthermore, the entry of another toxin called *granulysin* (a protease) persuades the target cell to undergo apoptosis. More significantly, destroying host cells by T cells is particularly important in preventing the replication of viruses.

Helper T cells

The activation of helper T cells can result in the release of cytokines as well as stimulation of macrophages such as killer T cells and B cells. The stimulation of B cells and macrophages induces a proliferation of T helper cells. These T cells regulate both the innate and adaptive immune responses and help the body to decide which kinds of immune responses to make toward a specific pathogen. It has been reported that these helper T cells have no cytotoxic activity and do not kill infected cells (Figure 5.9). Helper T cells express T cell receptors that identify antigens attached to Class-II MHC molecules. It has been reported that helper T cells have a weaker association with the MHC–antigen complex than killer T cells, which means many receptors located on the helper T cell can be attached by an MHC antigen to activate the helper cell. Moreover, helper T cell activation also requires longer duration of assurance with an antigen-presenting cell. In addition, helper T cell activation results in upregulation of molecules expressed on the T cell surface, which provide extra stimulatory signals normally required to activate antibody-producing B cells.

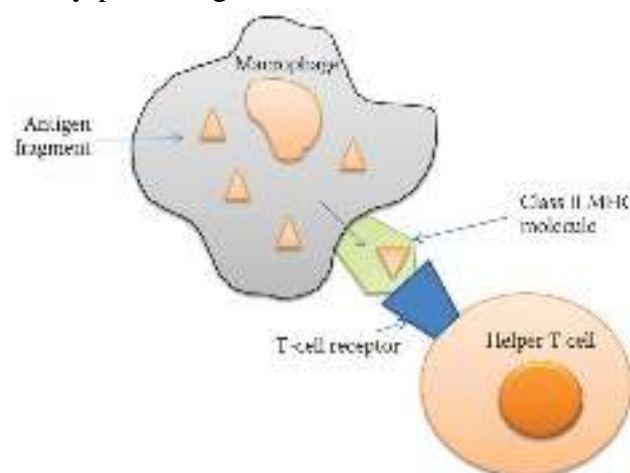


Fig-1 Function of T helper cells.

63-B lymphocytes and antibodies

The B lymphocytes and antibody cells detect pathogens when the antibodies on their surface attach to a specific foreign antigen. It has been described that this antigen–antibody complex is taken up by the B cell and processed by proteolysis into peptides. Later on, the B cells display these antigenic peptides on their surface of MHC Class-II molecules. As soon as B

cells are activated, they begin to multiply, and their offspring discharges millions of copies of the antibody that recognizes this antigen. Furthermore, these antibodies can circulate in blood and stick to pathogens expressing the antigen, destroying these pathogens with phagocytes.

Immunological memory

When B cells and T cells are activated and begin to reproduce, some of their offspring can become long-lived memory cells. This process is called immunological memory. It has been suggested that in all organisms, these memory cells can remember each and every specific pathogen encountered and can enhance a strong response if the pathogen is detected or enters again. This kind of immunological response is basically adaptive in nature, because it occurs during the lifetime of an individual. It has been reported that immunological memory can be classified as passive short-term memory or active long-term memory.

64-Passive memory

It has been reported that newborn infants have no prior exposure to pathogens and are particularly vulnerable to infections. During this critical period of child development, the mother provides immunity against any kind of infection by producing a particular type of antibody known as immunoglobulin-G, which is transported into the baby directly across the placenta. These antibodies help the infant to fight against any infection. Furthermore, mother's breast milk contains antibodies that are transferred to the infant and protect against bacterial infections until the newborn body can produce its own antibodies. This type of development of immunity between the mother and child is called passive immunity because the fetus does not make any antibodies on its own, it only borrows them from the mother. It has been reported that passive immunity is only for a short duration of time, up to several months. Interestingly, now, it is possible to induce protective passive immunity in the infant by artificial ways.

Active memory and immunization

It has been suggested that long-term active memory is acquired following infection by activating B cells and T cells. Active immunity can be produced by vaccination. The main principle behind vaccination is called immunization where a patient is injected with an antigen developed from a nonactive or killed form of pathogens to inspire the body to develop specific immunity against that particular pathogen.

65-Immune diseases

The human immune system is remarkable in protecting the body from illness; however, under disease conditions, the immune system may fail to defend the body against various infections. This is called an immune-compromised immune system. This condition makes the body vulnerable to various viral, bacterial, or fungal infections. The immune-related diseases can be classified into three broad categories such as immunodeficiency, autoimmunity, and hypersensitivities.

Immunodeficiency: Immunodeficiency is a physiological condition where the immune system's capacity to fight infectious disease has become dysfunctional or entirely absent. In most cases, immunodeficiencies are acquired diseases, but some people are born with defects in the immune system, which is known as a primary immunodeficiency. It has been reported that a person who has an immunodeficiency of any kind is considered to be immune-compromised and such a person may be particularly vulnerable to opportunistic infections, in addition to normal infections that could affect everyone.

Primary immunodeficiency

A number of rare diseases have been reported susceptible to infections from childhood onward and many of these diseases are hereditary and autosomal recessive or X-linked in nature. There are more than 80 primary immunodeficiency syndromes reported in humans and they are generally grouped by their malfunctioning of lymphocytes or granulocyte cells. The treatment of primary immunodeficiencies depends on the nature of the defect, and may involve antibody infusions, long-term antibiotics, and stem cell transplantation.

Acquired immunodeficiency

An immune deficiency that is caused due to external processes is called secondary or acquired immunodeficiency. The common causes for secondary immunodeficiency are malnutrition, aging, and particular medications such as chemotherapy, disease-modifying antirheumatic drugs, and glucocorticoids, which are immunosuppressive drugs after organ transplants. It has been reported that there are many specific diseases that directly or indirectly impair the immune system and these include many types of cancer, predominantly those of the bone marrow and blood cells, such as lymphoma, leukemia, multiple myeloma, and certain chronic infections. Immunodeficiency is also the hallmark of AIDS, caused by HIV.

66. Autoimmunity

In a healthy person, immunity generally protects the person from all infectious diseases, but in some individuals, it works against itself and destroys the person's immune system. This condition is generally known as an autoimmune disease. It has been suggested that any disease fallouts from such an abnormal immune response are called an autoimmune disease. Autoimmunity is frequently caused by a deficiency of germ cell development that results in the development of a condition where body cells work against their own cells and tissues. There are several examples of autoimmunity disease such as celiac disease, Churg–Strauss syndrome, Hashimoto's thyroiditis, Graves' disease, idiopathic thrombocytopenic purpura, diabetes mellitus type 1, systemic lupus erythematosus, Sjögren's syndrome, and rheumatoid arthritis (RA).

Classifications of autoimmune diseases

Autoimmune diseases can be classified into systemic autoimmune disease and localized autoimmune disorders, depending on the clinical and pathological features of each disease. The examples of systemic autoimmune diseases are Sjögren's syndrome, scleroderma, RA,

systemic lupus erythematosus, and dermatomyositis. It has been proposed that these disease conditions incline to be associated with autoantibodies to antigens, which are not tissue specific. The names of affected organs/tissues with autoimmune disease are listed in Table .1.

Table-1 Name of Syndromes That Affect Various Body Organs

Name of syndrome	Affected organ/tissue
Diabetes mellitus type 1	Pancreas
Celiac disease, pernicious anemia	Intestine
Pemphigus vulgaris, vitiligo	Skin
Autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura	Blood
Myasthenia gravis	Brain

67-Classifications of cell transplantation

In cell transplantation, the cells or tissues can be classified based on their utilization in different parts of the body. We have given a few examples where cell-based transplantation is effectively used in clinical research.

Autologous cells

The autologous cell transplantation is basically a transplantation in which cells obtained from patients can be transplanted into the same patient. One significance of the autologous transplantation is that there is a high rate of success as the transplanted cells easily can be accepted by the patient's body and there are few problems associated with ejection and pathogen transmission. There are however, a few shortcomings in the autologous cell transplantation as the availability of required cells to be transplanted are found in very low in numbers and also genetic disease-suitable autologous cells are not available. Another disadvantage of autologous cell transplantation is that older patients do not have sufficient numbers of autologous cells to be used for cell transplantation. The old patients need to undergo a surgical operation in order to isolate the cells which might lead to donor site infection or chronic pain. Autologous cells also must be cultured from samples before they can be used and these are lengthy procedures, so autologous cell transplantation might take a long time. In recent times there has been a trend toward the use of human mesenchymal stem cells (MSCs) isolated from bone marrow and fat cells. These MSCs can be differentiated into a variety of tissue types including bone, cartilage, fat, and nerve cells. A large number of cells can be easily and quickly isolated from a fat source, consequently opening the potential for large numbers of cells to be quickly and easily obtained.

68-Allogeneic cells

Allogeneic cells come from the body of a donor of the same species; for example, the cells from one person (donor) can be used by another person (patient). There are some ethical restraints on the use of human cells for *in vitro* studies, however, the use of dermal fibroblasts from human foreskin has been demonstrated to be clinically safe and consequently a viable choice for tissue engineering of skin.

Xenogeneic cells

The cells which are isolated from two different species are called as xenogeneic cells; for example, the cells from one species (sheep) can be used in another species (human). In particular, animal cells have been widely used in experiments aimed at the construction of cardiovascular implants which later can be used in humans.

Syngeneic or isogenic cells

This type of cell isolates from genetically identical individuals, such as twins, clones, or extremely inbred research animal models.

69-ORGAN TRANSPLANTATION

ORGAN TRANSPLANT SUCCESSES AND FAILURES

It has been over 50 years since the first successful human organ transplant was done—a kidney transplanted from one identical twin to another—and the procedure has become almost routine. The list of organs transplanted includes not just kidneys, but hearts, livers, lungs, and the pancreas. The number of transplants performed is impressive, considering both how complicated the surgery is and how strong a fight the immune system launches against the new organ. More than 325,000 transplants were performed in the United States from 1988 through August 2004. The average number per year is now about 25,000. Kidney transplants account for more than half of all transplants; liver transplants make up about 20%, hearts 10%, and lungs 5%. The number of transplants may seem large, but an even bigger number includes those people who are on a waiting list for an organ—more than 86,000 people right now.

Because human beings have two kidneys and can function just fine with only one, kidney transplants can come from living donors. In recent years, methods to transplant just a part of a liver have succeeded because the liver can repair and regenerate itself. A few hundred people each year, often children, receive a lobe of a liver from a family member.

Human-to-human organ transplantation has risks. The human immune system is finely tuned to recognize and destroy invaders, and because it carries different forms of transplantation markers from those of the recipient, the transplanted organ is seen as an invader, unless the donor and recipient share exactly the same genes, as in that first successful kidney transplant between identical twins. A major risk exists if antibodies to the donor cells are present in the recipient's blood at the time of transplant. If donor-reactive antibodies are present when the blood connection to the organ is made during surgery, the reaction against the new organ may

be so rapid that the blood vessels are blocked and the organ cells die. This rapid antibody reaction to the cells lining the organ blood vessels is called **hyperacute rejection**. Tests for antibodies lurking in the recipient's blood that could cause hyperacute rejection are performed before the transplant to determine who receives a donated organ. The ABO blood group system, critical in blood transfusion, is also important for some, but not all, transplanted organs. An organ donor must be a suitable ABO type for the patient or ABO antibodies in the recipient's blood will cause hyperacute rejection.

After the surgery, new antibodies and killer lymphocytes that cause rejection may develop within days. To prevent this from happening, or at least reduce the chances of it, scientists have discovered and developed a number of **immunosuppressive drugs** that help extend the life of the transplanted organ and, thus, the life of its recipient. **Corticosteroids** and the cancer chemotherapy drug azathioprine were the first drugs used to suppress the immune system for organ transplants. Although their long-term use can have serious side effects, steroids such as prednisone are still widely used. In the 1970s, the introduction of **cyclosporine**, the product of a soil fungus, dramatically improved one-year graft and transplant patient survival. Other widely used chemical immunosuppressive drugs include tacrolimus, mycophenolate mofetil, and sirolimus. Several monoclonal antibodies that remove or shut down T lymphocytes (such as muromonab, daclizumab, and basiliximab) are used to prevent or treat rejection. A preparation of animal antibodies raised against human lymphocytes is still used for the same purpose. Despite all these tools, slow or chronic rejection of the organ remains an ongoing problem. The success of organ transplantation is measured by organ and patient survival. Over 80% of kidney patients and 65% of their grafts survive for five years.

TAKING ORGANS FROM OTHER ANIMALS

Because the transplant waiting list continues to grow, scientists have explored nonhuman animals as a possible source of organs. Despite the shock and repulsion it sometimes causes, the idea of **xenotransplantation**, or transplantation of an organ from one species to another, deserves some serious consideration.

70-Transgenic organisms

Transgenic organisms are genetically modified organisms (GMOs) which have inserted DNA of different species origin. The inserted DNA/gene is known as transgene. Transgene is introduced into the organism, using recombinant DNA technology. The gene must be transmitted through the germline so that every cell, including germ cells, of the body contains the same modified genetic material.

Basic Requirements: In gene transfer, the knowledge of the biochemical and physiological mechanisms of its action, regulation of gene expression, and safety of transgene and its product is important. The process requires some basic steps for successful transfer:

- The extraction of nucleic acid (DNA/RNA). It is the first step in the genetic engineering process.
- After DNA extraction and selection of suitable gene, the next step is gene cloning . Gene cloning has four stages: (1) generation of DNA fragments, (2) ligation to a vector, (3) propagation in a host cell, and (4) selection of the transformed host.
- Gene is designed in such a way that it is under appropriate promoter and correct regulatory elements. Selectable marker gene may be incorporated for selection of the transformants from non-transformants.
- Incorporation of reporter gene allows detection of gene expression in transformed cells and enhancers are essential for efficient transcriptional control. 
- The gene of interest (GOI) is ligated to selectable marker gene and reporter gene. It is helpful for screening, detection, and identification of the transgene containing host cell. Reported genes may be β -glucuronidase gene (GUS) or green fluorescent protein or luciferase gene. GUS produces blue product when it acts on its substrate, and thus cells appear blue, whereas GFP and luciferase transformed cells emit light when excited with appropriate wavelength of light.

71-Production of Transgenic Animals

There are two basic strategies for producing transgenic animals. These are the so-called “gain of function” or “loss of function” transgenics. The “gain of function” relies on addition of a cloned fragment of DNA to an animal’s genome to obtain new expression of a gene product that did not previously exist in that cell or tissue type. “Loss of function” is a more direct way to determine the function of a lost/inactive gene after its alteration (Fig. 1) and then observe the physical manifestation (phenotype) of that genetic trait, for example, the mouse is obese or diabetic. These types of mice are often called knockouts, and from several years now, it has been possible to generate “ knockout” mice , which carry specifically defined mutations in the gene of interest using gene targeting in mouse embryonic stem cells .

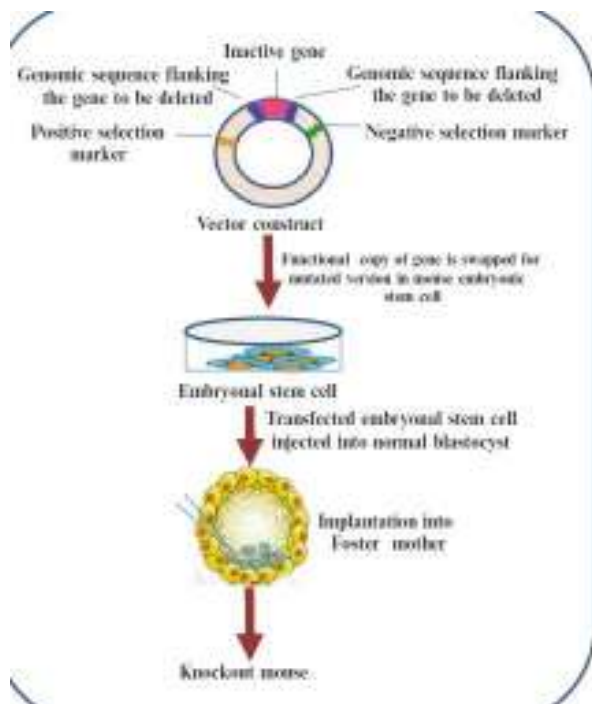


Fig-1 The figure shows the loss of function cassette where inactive gene is incorporated for homologous recombination with genomic sequence flanking the target gene with positive and negative selection marker gene. The vector exchanges itself in mouse embryonic stem cell for functional copy resulting in the production of knockout mouse.

72-Transfer of Transgene in the Animal

The essence of this technology resides in successful gene transfer in the intended animal and its insertion in the genome. The insertion of gene in the genome can be random or targeted. There are several methods of producing transgenic animals:

- (1) DNA microinjection,
- (2) retrovirusmediated gene transfer,
- (3) embryonic stem cell -mediated transfer, and
- (4) liposomemediated DNA transfer into cells and embryos.

A few modifications are also there for creating transgenics:

- Electroporation of DNA into the sperm, ova, or embryos and
- Nuclear transfer with somatic or embryonic cells.

73-Pronuclear Microinjection

Microinjection was the first technique used to generate transgenic mice by successful gene transfer using SV40 DNA directly into blastocoel cavity of preimplantation embryos and then their implantation in the foster mother. Eventually, the gene of interest may randomly integrate in the embryonic genome of some embryonic cells and ultimately contributes to germline. The technique was then extended to other animals (livestock). The method has poor efficiency and usually results in a high percentage of mosaics in which not all cells of the animal contained the transgene. The time and cost of screening for germline transmission from the major mosaic animals prohibits the generation of more transgenic animals. The method can also lead to high variability in transgene expression between animals due to

mosaicism, variable efficiency of transgene integration, and chromosomal position effects. The major advantage of this method is its applicability to a wide variety of species.

This method involves the direct injection of the gene construct into the cells to ensure guaranteed successful transfer of transgene to target cells. Presently the established technique involves the injection of DNA into the pronuclei of the fertilized egg. The host cell is held in position with a suction pipette and DNA is transferred into the nucleus through a fine needle. Thereafter the embryos with the transgene are cultured in vitro up to the morula stage and then transferred to pseudopregnant foster mothers. The insertion of DNA is, however, a complete random process; thus, there is high probability that either the introduced gene might be inserted into the host genome or, less commonly, it may remain extrachromosomal for one or more cell divisions. Thus, the resulting animal may be transgenic or chimeric for transgene insertion. Five to 40 % of mice developing from manipulated eggs contain the transgene. Once the transgene is transmitted through the germline, it tends to be stably inherited over many generations. The transgene insertion is accompanied by deletions and rearrangements occurring in genomic DNA. The limitation of microinjection is that it only allows for the random addition of exogenous DNA rather than targeting to specific sites. DNA targeting is necessary in generating gene knockouts, for instance, to model human diseases. The success of pronuclear microinjection is evident in the generation of transgenic pigs, goats, sheep, and cattle; but its limitations have hindered the progress of transgenics in livestock.

74-DNA Transfer Through Virus

Gene transfer mediated by means of a carrier or vector, generally, a virus or a plasmid, provides increased probability of expression. Virus has natural ability to stably introduce DNA to the host upon infection; therefore recombinant retroviruses and replication defective adenoviruses can be efficiently used as vectors. Retrovirus is efficiently capable of infecting dividing cells.

Initial attempts to produce transgenics by retroviral infection of early embryos invariably resulted in genetic mosaics caused by multiple insertion sites of the transgene. The short window of opportunity for the viral preintegration complexes to reach the embryonic chromatin during M-phase explains the delayed viral insertion, resulting in cell lineages with different insertion sites or no insertion at all. A significant advancement of this technique has been achieved recently by exposing metaphase II oocytes to transgenecontaining retrovirus. Arrested oocytes are particularly appropriate because they have undergone nuclear envelope breakdown and remain at metaphase II for a longer period compared with the M-phase of somatic cells (Fig.1). This maximizes the probability of preintegration complexes gaining access to the oocyte chromatin. The infected embryos are implanted in the uterus of foster mother to get germline transfer of transgene . Adenovirus represents an alternative to retrovirus as vectors to insert recombinant DNA into the mammalian germline.

However, the disadvantages of this technique are the possible interference of viral regulatory elements with the expression of genes and the susceptibility of virus to de novo methylation resulting in silencing of the genes. Though viruses are highly adapted to the process of gene transfer however, using virus as the vector may also produce health hazards as they may potentially arouse cancer/leukemia.

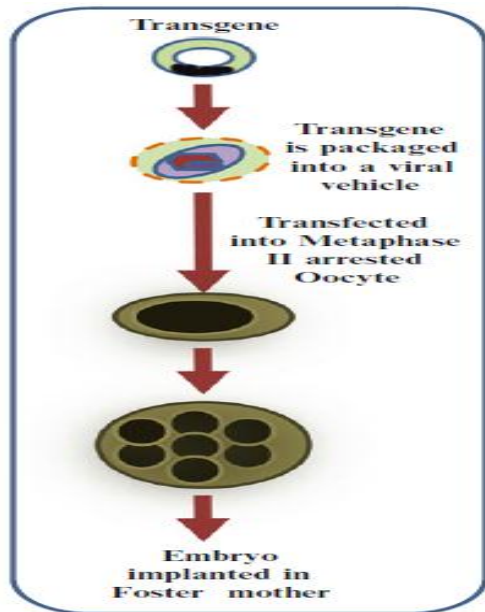


Fig-1 Virus-mediated transgenic production where the virus along with transgene is allowed to transfect metaphase II arrested oocyte. After transfection of oocyte, it is implanted in foster mother resulting in production of transgenic animal

75-Embryonic Stem Cell Mediated Gene Transfer

Embryonic stem cells are obtained from inner cell mass of blastocyst. This method involves prior insertion of the desired DNA sequence by homologous recombination into an in vitro culture of embryonic stem (ES) cells, and the transformed cells can be selected using standard markers. Cells containing the desired DNA are incorporated into the blastocoel of host's embryo at the blastocyst stage, where they mix with inner cell mass (Fig.1). Unlike the other two methods, which require live transgenic offspring to test for the presence of the desired transgene, this method allows testing for transgene at the cell stage. This is advantageous as there is no convenient way to select for eggs or embryos that have taken up foreign DNA.

The result is a true chimeric animal. ES cell-mediated gene transfer is the method of choice for gene inactivation, the so-called knockout method. This technique is of particular importance for the study of the genetic control of developmental processes. This technique works particularly well in mice. It has the advantage of allowing precise targeting of defined mutations in the gene via homologous recombination.

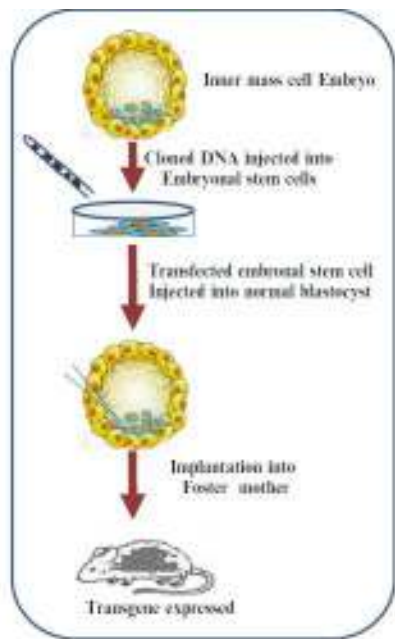


Fig- Embryonic stem cell-mediated gene transfer. The transgene is injected into the embryonal stem cells. These stem cells with transgene are injected into normal blastocyst, which is then implanted into the foster mother

76-Sperm-Mediated Transgenesis

The ability of sperm cells to carry exogenous DNA spontaneously into the oocyte during fertilization was first reported by Brackett and coworkers in 1971. However, the concept of sperm-mediated transgenesis rested for 18 years until scientists reported the use of spermatozoa as DNA carriers to produce transgenic mice. The scientific community was challenged by an unsuccessful attempt to replicate the experiment met this work with skepticism. However afterward transgene delivery by sperm cells was used to produce transgenic animals in a wide variety of species, including cattle, pigs, rabbits, frogs, and zebra fish. Sperm-mediated transgenesis is the most straightforward technique to produce transgenic animals. The spermatozoa are incubated with the DNA containing the gene of interest followed by in vivo or in vitro insemination.

DNA binds to the sperm's plasma membrane through specific DNA-binding proteins. After internalization, they are carried into the oocyte upon fertilization (Fig.1). The most appealing characteristics of using sperm as vectors to produce transgenic animals are its simplicity (no embryo manipulation is required) and the possibility of performing mass production of genetically modified animals through in vivo or in vitro insemination of many oocytes. On the other hand, this technique also has limitations.

Like pronuclear microinjection, no targeted modifications by homologous recombination could be achieved with this method due to the random integration of transgene. Recently scientists reported successful generation of transgenic pigs carrying a human gene (human decay accelerating factor, hDAF). In this study, approximately 80 % of pigs had integrated the construct containing an hDAF minigene; from this 80 %, approximately 53 % expressed the foreign protein at different levels. There is no doubt that this success represents an important step toward production of humanized pig organs and tissues for human

transplantation. If these findings are readily reproducible, sperm- mediated gene transfer may displace in short time the popular pronuclear microinjection method.

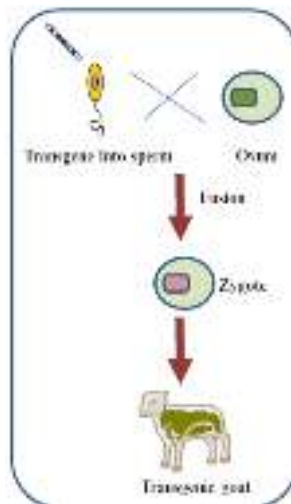


Fig-1 Sperm-mediated gene transfer where transgene is inserted into male pronucleus which upon fusion with ovum results in the production of transgenic goat

77-Somatic Cell Nuclear Transfer

Due to the absence of proven ES cells and the recent advances in nuclear transfer (NT), current emphasis for creating transgenic animals is on somatic cell nuclear transfer (SCNT). Nuclear transfer is a technique that is used to create a genetically identical copy, or a clone, of an animal. Nuclear transfer commonly involves the transfer or placement of a donor nucleus into the cytoplasm of an enucleated MII oocyte (Fig.1).

Donor cells can originate from a wide variety of cell types ranging from embryonic blastomeres all the way up to adult cells . Initial work on nuclear transfer was focused on using embryonic blastomeres as a donor source; the limited number of cells available in an early embryo hampered the process. Fetal or adult cells have been used successfully to clone all major livestock species, including sheep, cattle, goats, and swine. The ability to use cells where transgene can be introduced and which can be cultured increases the number of cells available to clone, thereby facilitating the ability to make transgenic animals.

Somatic cell nuclear transfer has facilitated the ability to make transgenic animals by circumventing most of the shortcomings of other transgenic techniques. First, the sex of the animal can be predetermined by choosing the donor material (i.e., male or female tissue). Second, the use of cell culture to propagate donor cells can lead to large numbers of transgenic cells that can be frozen and stored for long periods of time.

In conjunction with SCNT, these transgenic donor cells can eventually give rise to numerous cloned transgenic animals. Transgene structure and expression can be tested by molecular techniques, such as PCR , Southern blot analysis , fluorescence in situ hybridization, and Western blot analysis , before initiating nuclear transfer and transferring the embryo to a recipient cow with a lengthy gestation time of 9 months. The proper use of SCNT also ensures that 100 % of animals produced are transgenic and that every cell of a cloned animal

will have the transgene, thereby saving time and cost associated with recipient animals. The ability to use a clonal population of transgenic cells guarantees the same transgene insertion site for each clone, thus decreasing animal to animal variation in transgene expression levels. Further, transgene can be added to a specific genetic background. This technique can be used for the addition of DNA at random sites and targeted insertion of DNA by homologous recombination, which is vital in modulating specific gene expression as well as creating gene knockouts.

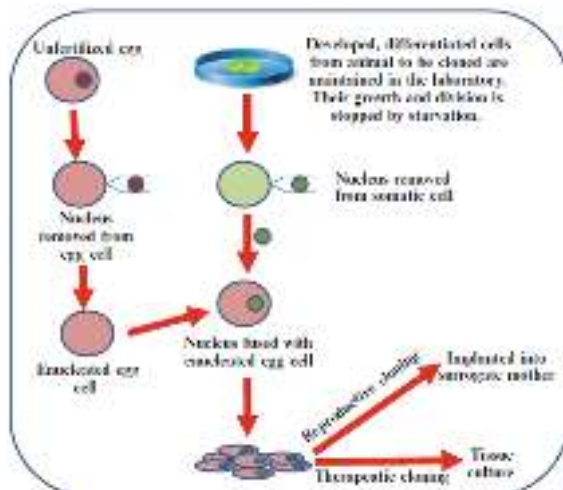


Fig-Somatic cell nuclear transfer where nucleus is removed from the egg cell and enucleated egg receives nucleus from donor somatic cell. The resultant egg with nucleus from somatic cell can be used for reproductive cloning or therapeutic cloning

78-Medical Applications of Transgenic Animals

In medicine the transgenic animals may serve as disease models, knockouts for studies related to gene function, quality trait improvement, and pharmaceutical production.

Transgenic Animals as Disease Model

Certain transgenic animals are produced for specific traits and serve as disease model. Animals are genetically manipulated to exhibit disease symptoms; thus they can be used for studies related to causes of the disease and effective treatment. For example, Oncomouse® developed by Harvard scientists is genetically modified and carries a gene that promotes the development of various human cancers.

Transgenic animals have enabled scientists to understand the role of genes in specific diseases. By either introducing or inactivating particular genes, researchers can discover the root causes of diseases associated with gene defects. Transgenic animals allow more effective treatments to be developed. Having found the genes implicated in a disease, scientists can then target these or design other therapies which act by influencing their expression.

Transgenic animals help test the safety of new medicines and vaccines. Because transgenic models can highlight specific characteristics such as certain mechanisms involved in the formation of tumors, they can demonstrate more clearly the possible side effects of new

therapies. Their use in early toxicity trials may also serve to prevent the subsequent use of a larger number of animals in the development phase.

79-Xenotransplantation

The gap between the demand for and the availability of human organs for transplantation is growing, a tendency that is not likely to reverse in the near future. The number of patients on waiting lists for organs has been growing every year, reflecting both an increased demand for organs for transplantation and a pronounced shortage of donors. Transgenic animals are being developed by some companies to provide new organs for transplantation such as kidneys, livers, and hearts. Pigs have long been considered as an alternative source of organs for xenotransplantation (i.e., transplantation of organs/tissues between different species, e.g., from animals to humans) to satisfy this increasing demand. Pigs seem to meet most of the requirements for an ideal animal donor: they are anatomically and physiologically similar to humans with organs of appropriate size, they are prolific, and they can be maintained under specific pathogen-free conditions. However, there are barriers to pig-to-human xenotransplantation; one of the main challenges is to avoid the hyperacute immunological rejection of the grafted tissue. When organs or tissues are transplanted between discordant species like pig to human, the host's immune system initiates a fast reaction known as hyperacute rejection (HAR).

80-Therapeutic proteins

One of the main discoveries in r-DNA technology was the production of biosynthetic human insulin, which was the first biomolecule made through r-DNA technology. Later on, this bioengineered insulin became the first biotechnology product approved by the U.S. Food and Drug Administration (FDA). It has been reported that insulin is basically an ideal candidate because it is a relatively simple protein and is therefore relatively easy to manipulate for genetic engineering. The first step in making recombinant insulin is the introduction of a specific gene sequence (oligonucleotide) that codes for insulin production in humans into the bacteria *E. coli*; it has been reported that only 0.94% of bacteria can pick up the sequence. Nevertheless, because the life cycle of *E. coli* is only about 30 min, there is no problem in generating a million copies of *E. coli* in a short period of time to be used for inducing insulin production. Among all applications of r-DNA technology, therapeutic proteins have been extensively used. Therapeutic proteins are those proteins that are either removed from human cells or engineered in the laboratory for pharmaceutical use. In addition to human insulin, other human proteins such as follicle-stimulating hormone, plasminogen, erythropoietin, and growth hormones have been created by using r-DNA technology (Figure 1).

There are many proteins essential to for the normal function of the human body and  unfortunately some people fail to produce sufficient amounts of these proteins in their body, which may lead to the development of functional deformities and genetic defects. These essential proteins include various blood-clotting factors causing hemophilia, insulin, growth

hormone, and other proteins. Patients who do not produce sufficient amounts of these proteins need to take these proteins externally either in the form of a medicine or an injection. These proteins can be synthesized outside of the human body by using recombinant technology. It has been reported that the majority of therapeutic proteins are recombinant human proteins manufactured using nonhuman mammalian cell lines that are engineered to express certain human genetic sequences to produce precise proteins of human use. Over the past decade, recombinant proteins are extensively used to replace deficiencies and to strengthen the immune system to fight cancer and infectious disease.

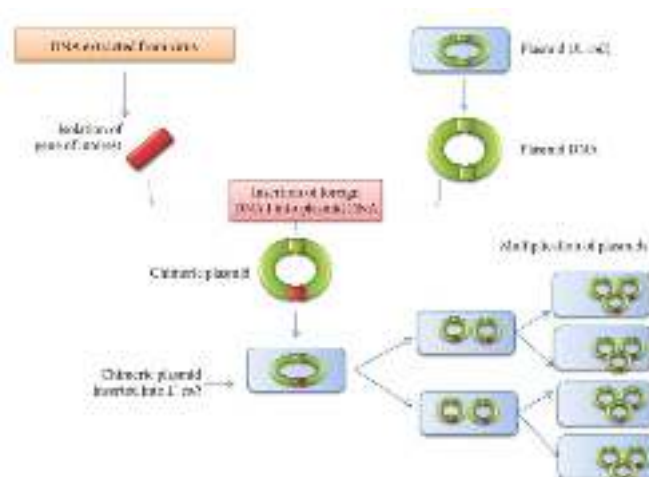


Fig-Making of chimeric proteins.

81-Recombinant human insulin

Insulin is a peptide hormone consisting of 51 amino acids. It is secreted by β cells of islets of Langerhans of pancreas. The hormone is responsible for maintaining normal blood glucose level in blood. Insulin is stored in the form of proinsulin which contains two polypeptide chains, A and B, and is connected with a third peptide C chain), which before secretion is cleaved with production of insulin and C-chain. The cleavage results in the removal of C-chain, and the A (21 amino acids) and B chain (30 amino acids) are linked by disulfide linkage to form mature insulin.

In the beginning, efforts were made to isolate mRNA for pre- and proinsulin from rat islets of Langerhans of pancreas and to synthesize cDNA. Thereafter, it was inserted into a plasmid. The recombinant plasmids were transferred into the *E. coli* cells, which secreted proinsulin. Scientists have chemically synthesized DNA sequences for two chains, A and B, of insulin and separately inserted into two pBR322 plasmids by the side of β -galactosidase gene.

The recombinant plasmids were separately transferred into *E. coli* cells which secreted fused β galactosidase-A chain and β -galactosidase-B chain separately. These chains were isolated in pure form by detaching from β -galactosidase with yields of about 10 mg/24 g of healthy and transformed cells. Production of recombinant insulin is shown in (Fig.1).

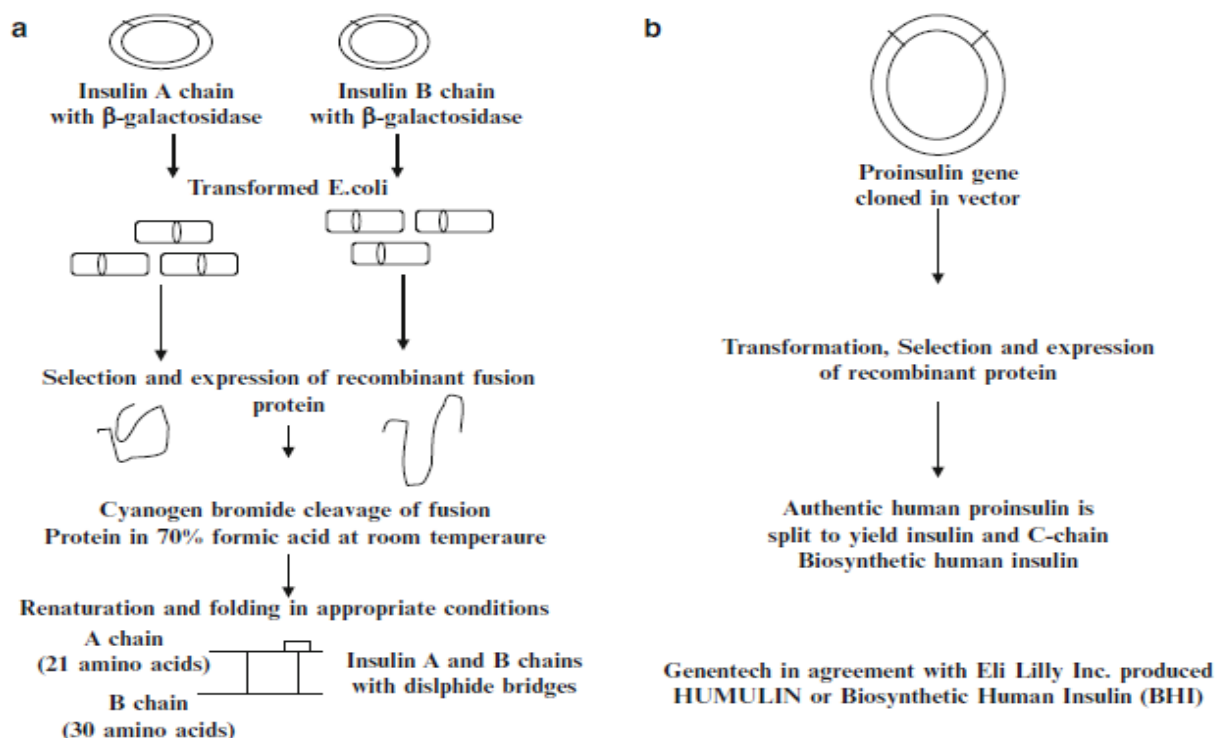


Fig-The figure shows the production of insulin. (a) The A and B chains of insulin are cloned separately in the vector. The transformation is done and after selection, the recombinant protein is obtained for both A- and B-chain genes. Cleavage by using cyanogen bromide removes the bacterial protein. The protein is renatured and provided suitable conditions for folding. (b) This figure shows the production of insulin from proinsulin gene which yields insulin and C-peptide. This is preferred method for production of biosynthetic human insulin.

82-Recombinant human growth hormone

Growth hormone is produced by the anterior lobe of the pituitary gland and is released in multiple pulses. The hGH is encoded by GHN gene cluster (an array of five closely related genes), which is localized on chromosome 17. It belongs to diverse gene family that has evolved by gene duplication events and has lots of structural similarity and some common functions. Of the various forms, the predominant form of hGH is 22 kDa protein of 191 amino acids with two disulfide bonds.

GH does not control the functions directly, but acts on certain hormones or somatomedins for its activity, for example, insulin-like growth factor 1 (IGF-1). It has a wide spectrum of roles to play as promotion of long bone growth, promotion of normal sex organ development through puberty, regulation of metabolism, stimulation of tissue growth and repair, anabolic/anti-catabolic effect via improved nitrogen retention, modulation of bone mineral density and metabolism throughout life, proliferation of some cell types of the immune system, appetite stimulation, and breakdown of fat (lipolysis). In clinical conditions, the therapy of growth hormone is given in the treatment of dwarfism, bone fractures, skin burns, bleeding ulcers, and AIDS.

Recombinant human growth hormone (rhGH) is 22 kDa consisting of long chain of amino acids. It is used in deficiency disorders of growth hormone. In children, it is used for growth abnormality as short stature and is used in chronic renal insufficiency. The therapy of growth hormone is also approved for adult growth hormone deficiency. GH is one of the most widely used hormones with the estimated market of more than 1.7 billion USD. Long experience in its administration has proven the therapy as safe and effective in various conditions of growth abnormality. Earlier pituitary-derived hGH was used but later on it was prohibited when found associated with Creutzfeldt–Jakob disease. Because of recombinant DNA technology, safe and abundant recombinant hGH was produced in various heterologous systems. As the non-glycosylated human growth hormone was biologically active, thus, the preferred system for its production is *E. coli*, which allows its rapid and economical production in large amounts.

Recombinant hGH (rhGH) is now used to treat:

- GH-deficient (GHD) short-stature children.
- Acceleration of wound healing.
- Increase in insulin-like growth factor (IGF)-1 levels.
- rhGH increases IGF-1, osteocalcin, type I procollagen pro-peptide (PICP), and bone density, when administered to children with GHD

For optimal productivity, strong inducible promoters are preferred as IPL, IPR, trc, and T7 in *E. coli*. They are advantageous as they drive overproduction of recombinant proteins. Apart from *E. coli*, human somatotropin (hST) expression was tried in a biologically active, disulfide-bonded form in tobacco chloroplasts. The hormone is used for the treatment of hypopituitary dwarfism in children; additional indications are in treatment of Turner syndrome, chronic renal failure, HIV wasting syndrome, and possibly treatment of the elderly. Growth hormone deficiency in human occurs both in children and adults.

83-Recombinant blood clotting factor VIII

Factor VIII is one of the important factors of all blood clotting factors. Deficiency of factor VIII causes bleeding disorder called hemophilia A. The hemophilia may be mild to severe depending upon factor VIII concentration in the body. In moderate and severe factor VIII deficiency, there can be spontaneous bleeding episodes in the joints. Hemophilia A affects 1 in 5,000–10,000 males. Replacement therapy is the treatment option for hemophiliacs either with human plasma-derived factor VIII (pdFVIII) or recombinant FVIII (rFVIII). Transfection of HEK 293 cell cultures in serum-free suspension is being tried for optimal yield. Recombinant factor VIII (rFVIII) is produced by culturing mammalian cells as baby

hamster kidney (BHK) or Chinese hamster ovary cells (CHO), using large-scale bioreactors . Standardizations are done to maximize yields.

84-Recombinant tissue plasminogen activator

Tissue plasminogen activator (tPA) is another kind of serine protease protein that is involved in the breakdown of blood clots in humans (Figure 1). In the human body, tissue plasminogen is found on endothelial cells, the cells that line the blood vessels. As an enzyme, tPA catalyzes the conversion of plasminogen to plasmin, the major enzyme responsible for clot breakdown. It has been reported that tPA is used as clinical medicine to treat only embolic or thrombotic stroke. Patients who have a problem related to blood clotting need a medicine to clot the blood immediately to avoid further releases of the blood from the body and tPA is considered to be an important medicine to promote blood clot. The tPA can be manufactured by using r-DNA technology and the production of tPA created this way is referred to as recombinant tissue plasminogen activator (rtPA).

The basic principle behind blood clotting is that tPA catalyzes the conversion of plasminogen into plasmin, which is done by cleaving the single-chain plasminogen into two chains. These two chains are connected by a disulfide bond and the resulting molecule is called plasmin. In the patients, an increased enzymatic activity can cause hyper fibrinolysis, which demonstrates as an excessive bleeding. tPA is used in various diseases such as pulmonary embolism, myocardial infarction, stroke, and thrombolysis. The first step in any kind of blood-clot-related disease is to stop the outflow of the blood, so the first thing any physician will do is to immediately inject tPA within the first 3 h of the event or within 6 h to be able to clot the blood. As per the guidelines used in Canada, hospitals for ischemic strokes suggest that tPA must be given within 4.5 h of the onset of symptoms, and because of this, only about 3% of patients were able to qualify for this treatment, as most patients do not seek medical assistance quickly enough. With regard to the situation in the United States, the window of the administration of tPA is 3 h from the onset of symptoms.

There are various commercially available rtPAs in the market, which include Alteplase, Reteplase, and Tenecteplase. It has been reported that Alteplase is an FDA-approved drug for the treatment of myocardial infarction, acute ischemic stroke, and acute massive pulmonary embolism. Reteplase is also an FDA-approved drug for the treatment of acute myocardial infarction and it has more convenient administration and faster thrombolysis than Alteplase. Consequently, Tenecteplase is also specified in acute myocardial infarction, and it showed fewer bleeding complications. There are other types of tPAs are under trial phases, including Desmoteplase. One of the major concerns with regard to tPA treatment is that tPA quickly dissolves blood clots, which might lead to a risk of hemorrhage in some patients. Considering the fact that the use of tPA may cause hemorrhage, there are alternative medicines available in the market that do not cause any hemorrhage. One example is streptokinase, which is a cheaper alternative that can be used as a thrombolytic in acute treatment.

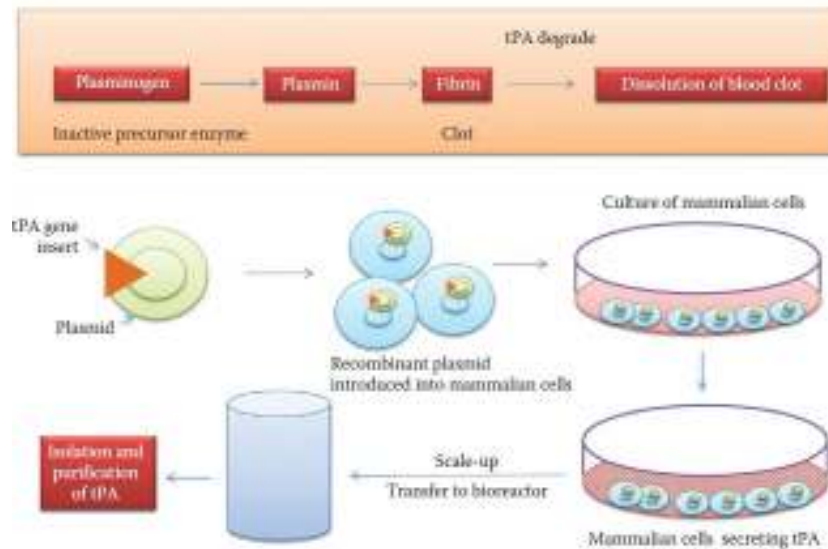


Fig-1 Making of recombinant tissue plasminogen activator.

85-Recombinant erythropoietin

Like insulin, erythropoietin (EPO) is a glycoprotein hormone that controls red blood cell production in the body (Figure 1). EPO, also called hemopoietin, is known to be located as precursors in the bone marrow. It is produced by the peritubular capillary endothelial cells in the kidney and liver. Besides its role in red blood cell production, EPO also plays an important role in neuronal injury and in the wound healing process. It has been reported that EPO has its primary effect on red blood cells by protecting the blood cells from going to the apoptosis pathway. It also conjoins with various factors involved in the growth of precursor red cells. EPO has a range of actions in hypertension, stimulating angiogenesis, and inducing proliferation of smooth muscle fibers. It has also been shown that EPO can upsurge iron absorption by quashing the hormone hepcidin.

Applications of erythropoietin

There are various applications of EPO. EPO can be used as a therapeutic agent to treat various disease conditions. It can be used in the treatment of anemia resulting from chronic kidney disease and myelodysplasia, and in the treatment of cancer. There are various types of EPO produced for patients who are suffering from anemia. These are epoetin, darbepoetin, epoetin delta, PD poetin, and methoxy polyethylene glycol-epoetin beta. The list of erythropoietin produced by using r-DNA technology is given in Table -1.

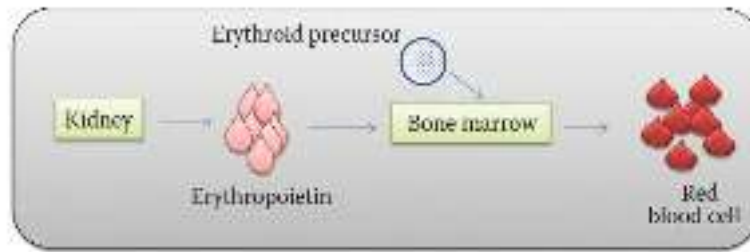


Fig-Normal synthesis of red blood cells

Table-List of Erythropoietin Produced by r-DNA Technology

Trade name of erythropoietin	Name of manufacturer
Epogen	Amgen
Espogen	LG Life Sciences
Epotin	Gulf Pharmaceutical Industry (Julpfar)
Betapoeitin	CinnaGen and Zahravi
Erykine	Intas Biopharmaceutical Pvt. Ltd.
Shanpoeitin	Shantha Biotechnics Ltd.
Zyrop	Cadila Healthcare Ltd.

86-Significance of vaccines

A vaccine is a biological preparation that improves human immunity to a particular disease. Moreover, a vaccine basically contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microorganism or its toxins. Upon injecting these vaccines in humans, the vaccine molecules stimulate the body's immune system to recognize the agent as foreign, destroy it, and memorize it, so that the immune system can more effortlessly familiarize with it and destroy any of these microorganisms that it later encounters. The story behind vaccine development started with the research work done by Edward Jenner in the late 1700s. Jenner took pus from the hand of a milkmaid with cowpox, inoculated an 8-year-old boy with it, and 6 weeks later variolated the boy's arm with smallpox, afterwards observing that the boy did not catch smallpox. Later on, more experimentation was performed to demonstrate the efficacy of the procedure in infants. Interestingly, Louis Pasteur generalized Edward Jenner's idea by developing what he called a rabies vaccine, now termed an antitoxin, which caused an extensive development of vaccines around the globe and obligatory vaccination laws were passed. Furthermore, researcher Maurice Hilleman was the most successful developer of vaccines in the twentieth century. Vaccines became more common among all populations of the world; however, vaccines remain elusive for many important diseases, including malaria and HIV.

Type of vaccines

Vaccines are produced by either dead or inactivated organisms and there are four main ways to develop vaccines:

- (i) live attenuated vaccines contain bacteria or viruses that have been altered so they cannot cause disease,
- (ii) killed vaccines contain killed bacteria or inactivated viruses
- (iii) toxoid vaccines contain toxins (or poisons) produced by the germ that have been made harmless
- (iv) component vaccines contain parts of the whole bacteria or viruses.

87-Live attenuated vaccines

The live attenuated or weakened vaccines can be created from the naturally occurring microorganisms; however, microorganisms used in this vaccine development can still infect people, but they rarely cause severe disease. Viruses are weakened by growing them over and over again in cell culture conditions. Furthermore, the procedure of growing a virus under cell culture conditions is known to decrease their disease-causing capabilities. There are various examples of live attenuated vaccines that include measles vaccine (as found in the MMR vaccine), rubella (German measles) vaccine (MMR vaccine), oral polio vaccine (OPV), mumps vaccine (MMR vaccine), and varicella (chickenpox) vaccine.

The advantages of attenuated vaccines are:

- Single dose is effective, and boosters are not essentially required because immune system is exposed to the live organism for prolonged period.
- Efficiently activates adaptive immune responses.

Disadvantages:

- Storage conditions are to be strictly maintained.
- Reversion is one of an important risk.
- Vaccines can cause serious complications in immunocompromised patients

88-Inactivated vaccines

Inactivated vaccines cannot cause an infection, but they still can stimulate a protective immune response. Due to problem of reversion of pathogenic agent in attenuated vaccines, the live virus/bacteria were inactivated by heat or chemical treatment. Viruses can be inactivated with chemicals such as formaldehyde. These inactivated vaccines have microorganisms incapable of replication but is able to activate immune system for memory response. Controlled inactivation is done for production of vaccine where microorganism is properly inactivated (that is killed), but at the same time, care is taken to maintain the surface structure of epitope (for specificity and memory). Heat inactivation is not preferred because it causes denaturation and thus loss of native structure of the epitopes. Chemical inactivation using formaldehyde or various alkylating agents has been successfully used. In contrast to

attenuated vaccine where one dose is sufficient for long term immunity, inactivated killed vaccines require boosters with appropriate adjuvants for activating immune system of the host. The humoral immune activation is the predominant immune system branch, which responds for inactivated/ killed vaccines. One example of an inactivated vaccine is the inactivated polio vaccine (IPV), which is the shot form of the polio vaccine, inactivated influenza vaccine.

The complications of inactivated vaccines were observed when Salk vaccine for polio was prepared and formaldehyde failed to kill the entire virus in two lots resulting in outbreak of paralytic polio in a high percentage of recipients.

89-Toxoid vaccines

There are some kinds of vaccines that can be made by treating them with toxins (formalin) produced by microorganisms, to destroy their ability to cause illness. Additionally, toxoids do not cause disease; in fact, they stimulate the body to produce protective immunity. There are several examples of toxoid-based vaccines such as diphtheria toxoid vaccine, which may be given alone or as one of the components in association with DTP, and furthermore, tetanus toxoid vaccine can be given alone or as part of the DTP.

Component vaccines

Some vaccines are made by using only parts of the viruses or bacteria. These vaccines cannot cause disease; nevertheless, these vaccines can stimulate the body to create an immune response that protects against viral infection with the whole germ. So far, four vaccines have been produced by using this method that includes hemophilic influenza type b (Hib) vaccine, hepatitis B (Hep B) vaccine, hepatitis A (Hep A) vaccine, and pneumococcal conjugate vaccine.

90-DNA Vaccines

DNA vaccines came into being in 1990. Since that time they have been hailed as a novel approach to DNA technology. Entire scientific meetings are devoted to DNA vaccines, an Internet site has been created to disseminate germane information, and according to the respected journal Science "... biotech companies are tripping over each other to get into the business..." of producing DNA vaccines. DNA vaccines consist of plasmids engineered to contain a protein-encoding gene and all the necessary requisites. Unlike replicating viruses or live bacteria, they are not infectious or replicative, nor do they encode any proteins other than those specified by the plasmid genes. Thus, one of the outstanding features of the DNA vaccine is its safety. But a DNA vaccine is not simple: A single plasmid might contain a promoter site, a convenient cloning site for the gene of interest, a poly-A tail used as a termination sequence, an origin of replication, and a selectable marker sequence such as an ampicillin-resistant gene (in addition, of course, to the protein-encoding gene or genes).

One highlight of the DNA vaccine is that no special formulation is necessary. Animals can be immunized against disease merely by injecting plasmids suspended in saline (salt) solution. And delivery of the plasmids to target cells is relatively easy: as early as 1994, researchers

identified five effective ways for delivering influenza virus plasmids to the animal body: by nasal spray; by injection into the muscle, vein, and under the skin; and by a so-called gene gun. The latter is a propulsion device that shoots DNA-coated gold beads into the skin. All techniques resulted in protective immune responses.

Furthermore, deploying plasmids as DNA vaccines has the added advantage of stimulating both antibody-mediated immunity (related to B-lymphocytes) and cell mediated immunity (related to T-lymphocytes). This is because DNA vaccines encode proteins that are released from the cell and taken up by phagocytes for delivery to the B-lymphocytes for antibody production. At the same time, some of the proteins fix themselves to the target cell surface and stimulate a response by T lymphocytes.

Later when the pathogen appears, the antibodies and T-lymphocytes will still be available to neutralize the pathogen's proteins and destroy the pathogen itself (or the cell harboring the pathogen). DNA vaccines also appear to elicit a higher immune response than scientists anticipated, despite the tiny amount of antigenic protein encoded. Researchers guess that this added benefit is due to an immune response to the other genes contained in the plasmid. The products encoded by these genes, like the main gene products, are interpreted as foreign and "...increase the wallop of a DNA vaccine," as one writer has said.

Even the production of DNA vaccines is advantageous. The technological methods for producing the plasmids are now in place, and biochemists can adapt their methods for various DNA vaccines. By using similar fermentation, purification, and validation techniques to produce a spectrum of DNA vaccines, manufacturers can reduce time and cost. Moreover, DNA vaccines are more stable at low and high temperatures compared with conventional vaccines. They can be shipped easily and inexpensively and reach a greater population of vaccinees. At this writing, DNA vaccines have been used experimentally to protect against influenza, *Salmonella typhi*, HIV infection, herpes simplex, hepatitis B, and several cancers including melanoma, colon cancer, renal cell carcinoma, and T-cell lymphoma.

Public health specialists envision that using DNA technology, vaccines can be developed quickly against emerging diseases (e.g., those caused by Ebola virus and drug-resistant bacteria), and they foresee that a number of different diseases can be controlled with a single vaccination by simply adding more genes to the plasmid.

There are drawbacks to consider, however. The DNA vaccines do little to address pathogenic mechanisms involving carbohydrates (as in streptococcal pneumonia, for instance). Delivery systems must be worked out, and ages determined for optimum immune response. The vaccines must also be evaluated for therapeutic uses in already infected patients and in those with chronic conditions.

Over the years, the research with DNA vaccines has been modified and expanded for various purposes. One approach has been to induce the body's B-lymphocytes to produce both antigens and antibodies. In 1997, researchers Stephen Johnston and Maurizio Zanetti at the University of California described how they induced a mouse's B-lymphocytes to perform double duty: The researchers injected the B-lymphocytes with plasmids containing genes

from malarial parasites and genes that encode antibodies against malarial parasites. Soon the spleen cells were producing antibody molecules bound to antigens from the malarial parasite. The antibodies reacted with the malarial parasite causing disease while the antigen portions stimulated other B-lymphocytes to produce still more antibodies. The "antigenic antibody" is an extremely novel idea in immunology.

Researchers at the University of Texas have been equally imaginative as they attempt to identify which plasmids are the key to defense against a pathogenic organism (in effect they hunt for the plasmid giving the strongest immune response).

The scientists take a pathogenic organism's DNA and break it to fragments. Then they prepare perhaps 1000 different plasmids. Various plasmid groups are injected to animals as DNA vaccines, and the animal is given time to develop an immune response. Then the pathogen is injected. If the animal remains unaffected by one of the plasmid groups, the key element for protection is in that group. The group is subdivided into smaller groups and the test is repeated. Again, the uninfected animal points to the smaller plasmid group. Retest after retest follows, and the scientists eventually reach a single or few plasmids that are conferring protection. Those are the ones presumably useful for a DNA vaccine.

The fresh approaches to vaccine research bode well for future generations and represent an elegant application of the DNA technology. Scientists have had difficulty restraining their enthusiasm for the new vaccines. But that is not unusual because they have had difficulty restraining their enthusiasm for most prospects of DNA technology.

The advantages are:

- Protein is expressed in its native conformation.
- Long-term exposure of immune system to the antigen.
- Efficient activation of cell-mediated and humoral immune systems leading to memory response.
- There is no requirement of specific storage condition like refrigeration of plasmid DNA, and it reduces the cost and is easy to handle.
- The genes expressing cytokines can be ligated to the vector, and the cytokine produced at the site may activate the desirable branch of immune system.
- Vaccine is safe, without any infectious agent.
- Easier to produce in large quantity.

The disadvantage of DNA vaccine is that they can be used for only protein antigens, and for the non-protein antigens (polysaccharide) they cannot be used.

91. Nano Vaccine

A vaccine is a biological preparation that improves immunity to a particular 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 or its toxins. The agent stimulates

the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters.

Nano vaccine: Nano is very vast field and it can be applied to any area, one of such area is vaccine. It provides a different routes of administration of vaccine. Nano vaccine can be designed, manufactured and introduced into the human body to improve health, including cellular repairs at the molecular level. The nanomaterial is so small that it can easily enter the cell; therefore, nanomaterials can be used in vivo or in vitro for biological applications.

Delivery of Nano-vaccine

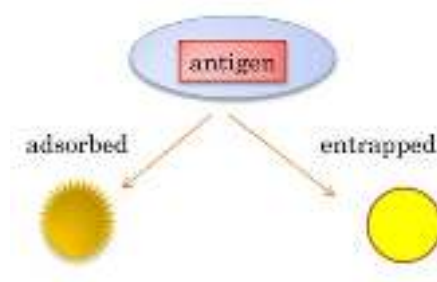
Following are the different way to deliver vaccine at nano level

1. Nanobead: Inert solid bead Size range 20-200nm.

- When antigens are adsorbed on the surface of bead it has been shown to stimulate CD8 -T cell response.
- The size of the bead play a major role in eliciting a combined response of humoral and cell-mediated immunity.
- Antigen covalently linked to inert nano-beads with a size of ~50 nm is preferentially taken up by dendritic cells, thus inducing humoral as well as cell-mediated immune responses.

2. Polymeric nanoparticles

- Biodegradable, biocompatible polymers have been approved for use in humans.
- Poly(D,L-lactide-co-glycolide) (PLG) and polylactide (PLA).



- Polyglycolide (PLG) have been extensively used to encapsulate antigens.
- PLG forms lactic and glycolic acids, After hydrolysis of α -hydroxyl acids, yielding small spherical polymeric particles 1–100 nm in size.
- Adsorbed antigen offer improved stability and activity over encapsulated antigen by avoiding exposure to organic solvents used during formulation and acidic pH conditions caused by degradation of the polymer.

3. Nano-emulsion:

- Size of globule (100-400nm)
- Nano-emulsion vaccine does not require refrigeration and is stable for 6 months.
- Nano-emulsion is non-toxic, pain free and avoids the risk of spreading needle-borne infections.
- Nano-emulsion of hepatitis B antigen, has been reported to be a safe and effective hepatitis B vaccine.

LECTURE 92:-

ADJUVANTS: These substance used in combination with a specific antigen that produced a more robust immunity than the antigen alone. It improve the immune response to vaccine antigen by different way:

1. Increasing immunogenicity of weak antigen.
2. Enhancing the speed and duration of the immune response.
3. Modulating antibody avidity, specificity, isotope or subclass distribution.
4. Examples
5. MF59
6. Monophosphoryl lipid A
7. Montanide™
8. Calcium phosphate nanoparticles.
9. Immunostimulating complexes
10. Cholesterol-bearing hydrophobized pullulan
11. Nanoparticles (CHP)

MF59: MF59 is the only nano-sized vaccine adjuvant approved for human use. MF59 is an oil-in-water emulsion (≤ 250 nm droplets). Its showed a 34-fold increase in antibody titers when immunized with glycoprotein D of herpes simplex virus (HSV) in guinea pigs. The mechanism of adjuvanticity of MF59 is believed to be through direct stimulation of cytokine production.

Monophosphoryl Lipid a: Monophosphoryl lipid A (MPL®) is an immunostimulant TLR-4 receptor agonist composed of detoxified lipopolysaccharide (LPS) from Salmonella Minnesota R595. It is a versatile vaccine adjuvant that may be included in aqueous formulations or in an oil-in water emulsion for a more dynamic response.

Montanide™: There are several different types of Montanide™, including:

- ISA 50V, 51, 206 and 720.
- ISA 50V, 51 and 720 are water-in-oil emulsions while ISA 206 is a oil-in-water emulsion.
- ISA 206 and 50V only used in veterinary vaccine formulations.
- SA 51 & ISA 720 are under investigation for use in humans.

Emulsions of Montanide™ ISA 51 and 720 are composed of a metabolizable squalene-based oil with a mannide monooleate emulsifier. The immune enhancement produced by the Montanide™ emulsions is believed to be due to the formation of a depot at the site of injection. The emulsion vaccines against malaria, HIV and various cancer have been in phase I and/or II clinical trials. A phase I trial of a trivalent malaria vaccine containing ISA 720 induced both humoral and cellular immune responses

Calcium Phosphate Nanoparticles

Calcium phosphate nanoparticles are less than 1000nm in diameter. Nanoparticles can be generated by combining (while stirring) calcium chloride, sodium phosphate and sodium citrate. It produces longer duration of response as compare to Al salt. Phase I study showed that CaP is safe and non-toxic when administered subcutaneously. Vaccines utilizing CaP in preclinical studies include anthrax, HBV, flu (H5N1 avian and seasonal) and HSV-2.

Advantages of Nano-vaccine

- Nano-vaccine have potential to deliver safe and more effective vaccine.
- Nanobead covalently coupled with antigen offer distinct advantages – a low dose of antigen is required, efficient processing by antigen-presenting cells and stability during storage.
- Encapsulated nanoparticles easily deliver antigen, protects the antigen from degradation and is found to be effective with a single dose due to slow release of the antigen.
- Many of the nano-vaccines are non-invasive, delivered by the oral or nasal route, diffusion patches or microneedle arrays, thus allowing pain-free delivery with minimal damage. This is an advantage over conventional vaccines, which are usually multi injection, multi-dose delivery systems.

Disadvantages

- Cost of production.
- Nanomaterials can change size, shape but not composition, which may change their toxicity.

- Small nanoparticles are cleared quickly from the body, large counterparts may accumulate in vital organs causing toxic problems. 

LECTURE 93:-

NANOPORE SEQUENCING

DNA Sequencing: DNA sequencing is the process of determining the precise order of the nucleotides within a DNA molecule. DNA sequencing may be used to determine the sequence of individual genes, larger genetic regions, full chromosome or entire whole genome.

What is Nanopore?

- Nanopore = Very small hole
- Electrical current flows through the hole
- Introduce analyte of interest in the hole
- Identify analyte by the disruption or block to the electric current.

Introduction

The fourth-generation DNA sequencing technology. Studies the interaction between DNA and protein, as well as between protein and protein. Have the potential to quickly and reliably sequence the entire human genome for less than \$1000, and possibly for even less than \$100. The detection principle is based on monitoring the ionic current passing through the nanopore as a voltage is applied across the membrane. When the nanopore is of molecular dimensions, passage of molecules (e.g., DNA) cause interruptions of the current level, leading to a signal.

Since DNA bases (adenine, thymine, cytosine and guanine) are different from each other in atomic scale, it is essential to collect base-specific information at atomic level to correspond the DNA sequence with the measured signals.

According to the different types of the signals, the detection methods can be roughly classified into two categories:

- The electrical detection methods and the optical readout method.
- In the 1990s, Church et al. And Deamer and Akeson separately proposed that it is possible to sequence DNA using nanopore sensors

Types of Nanopores Sequencing

Nanopore technologies can be broadly divided into two categories:

1. Biological nanopore

2. Solid-state nanopore

More recently, hybrid nanopores have been proposed to take advantage of the features of both biological and solid-state nanopores.

LECTURE 94:-

BIOLOGICAL NANOPORE SEQUENCING

Biological Nanopores

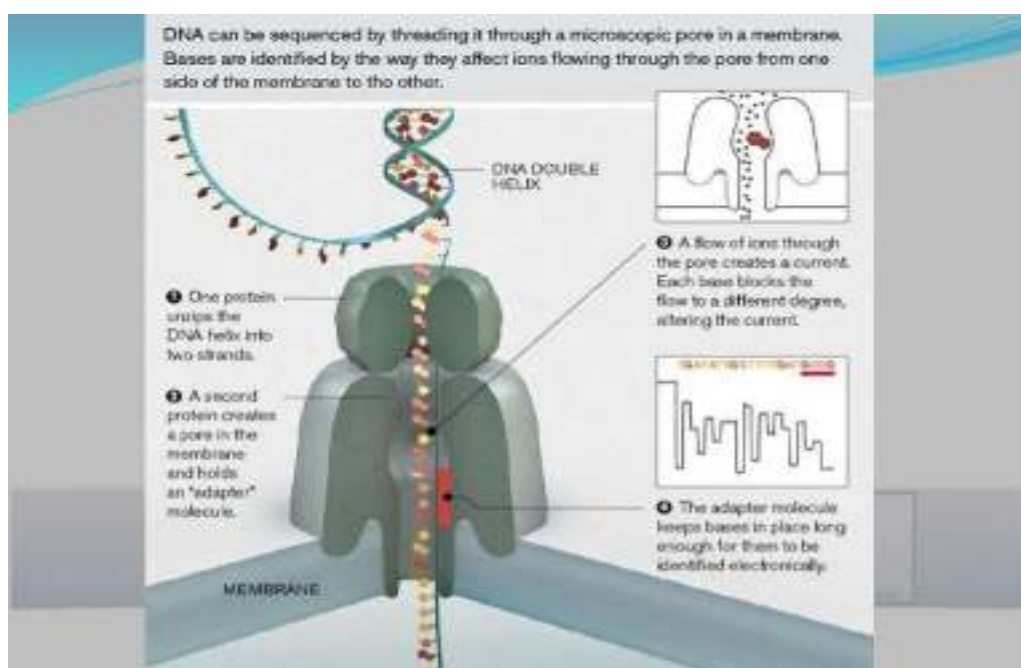
Also called transmembrane protein channels. Are usually inserted into a substrate, such as planar lipid bilayers, liposomes, or other polymer films. Nanopores formed by pore-forming proteins. Examples:

- alpha-hemolysin
- MspA
- phi 29

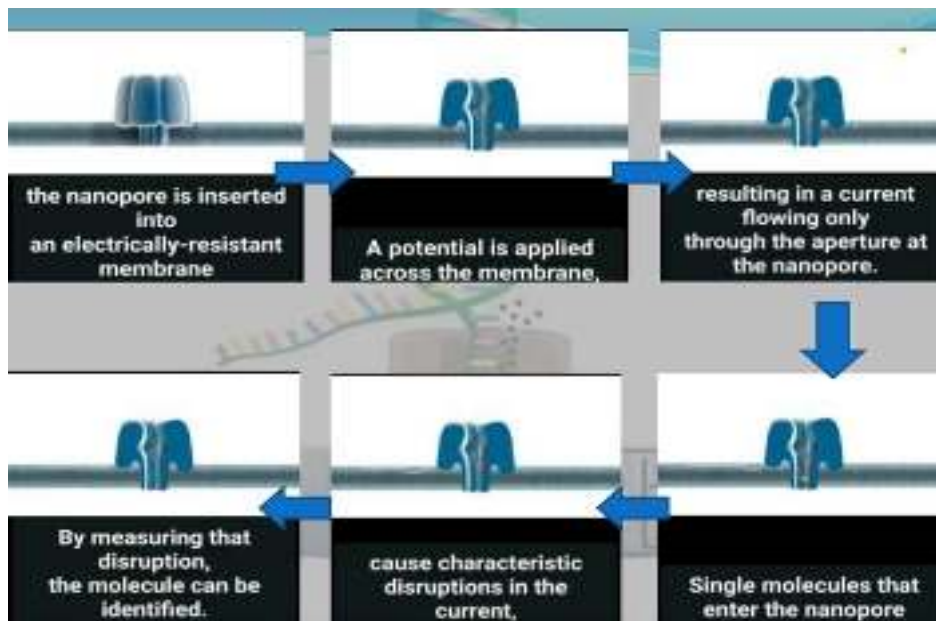
ALPHA-HEMOLYSIN: α -Hemolysin (α -HL, also called α -toxin). α -HL is an exotoxin secreted by the bacterium *Staphylococcus aureus*, a human pathogen. This mushroom-shaped heptameric protein normally forms pores in the membranes. The limited pore size (1.4 nm) has restricted its application in the analysis of ssDNA, RNA, or small molecules.

Mycobacterium smegmatis porin A (MspA): Powerful nanopore for reading information from four nucleotides simultaneously. By Butler et al. in 2008. The channel of the MspA octamer is 1 nm in diameter at the minimal point, which is relatively small and narrow, compared to that of α -HL. Thus, it can improve the spatial resolution of ssDNA sequencing

Principle



Working Process



Advantages of Biological Nanopores

- Their well-defined and highly-reproducible nanopore size and structure.
- Biological nanopores can be modified easily with modern molecular biology techniques, such as mutating the nucleotide sequence to change the amino acid residue at a specific site

Disadvantages of biological nanopores

- The environmental demands (like temperature, electrolyte concentration and Ph) of nanopores to keep their biological activities.
- The fragility of lipid bilayer makes the biological nanopore breakdown easily

LECTURE 95:-

Biopharmaceuticals: Biopharmaceuticals are defined as pharmaceuticals manufactured by biotechnology methods, with the products having biological sources, usually involving live organisms or their active components. A protein or nucleic acid used as a drug, which has been produced by living organisms as a result of biotechnology

Included products like

- Monoclonal antibodies
- Recombinant proteins or oligonucleotides used as vaccines
- Nucleotide sequences and vectors used in gene therapy
- Wide range of recombinant proteins used as drugs.

Excluded Products

- Proteins and nucleic acid extracted from sources that have not been manipulated using biotechnology e.g.
- Recombinant human growth hormone
- Growth hormone extracted from pituitary gland.

Pharmaceutical Technology

The methods and techniques that involve the use of living organisms (such as cells, bacteria, yeast and others) are tools to perform specific industrial or manufacturing process are called biotechnology. Pharmaceutical Technology will continue to provide new breakthroughs in medical research in the years to come, leading to treatment in field which have previously eluded us (including AIDS, cancer asthma, Parkinson's disease, Alzheimer disease)

The range of Biopharmaceuticals

First human recombinant protein somatostatin expressed in E.coli.....1970s

True biopharmaceutical produced was recombinant protein insulin...1982

First therapeutic use of monoclonal antibody was in 1986.

- Hormones
- Clotting factors
- Cytokines
- Growth factors

Thrombolytic factors and enzymes etc. Rapid increase in growth of biopharmaceutical products is due to combination of Increase in our understanding of disease processes. The relative ease with which same technique can be used to produce biologically active molecules.

Advantages of Biopharmaceuticals

- BP can be engineered to be identical to human proteins e.g. insulin
- Molecularly engineered biopharmaceuticals avoid the need to extract human protein thus avoiding transmission of diseases.
- BP are easy to produce (in lab) and less amount is sufficient.
- High degree of specificity i.e. limited to a certain species

Disadvantages of Biopharmaceuticals

- Large scale production is expensive that's why these are expensive drugs.
- Generic products are as expensive as new conventional drugs.

- Risk of contamination from infectious agents, cells or products
- Very small changes can produce significant changes in the product.
- An ideal drug is one which is given orally and one dose is enough unlike BP products.
- Despite their extreme selectivity these drugs do produce side effects.

LECTURE 96:-

Biopharmaceuticals are defined as pharmaceuticals manufactured by biotechnology methods, with the products having biological sources, usually involving live organisms or their active components. A protein or nucleic acid used as a drug, which has been produced by living organisms as a result of biotechnology

Large scale Production of Biopharmaceuticals

Most commercial production of biopharmaceuticals uses cultured mammalian cells. Other expression systems are

- Bacteria
- E.coli and Bacillus subtilis
- Simple physiology
- Grow rapidly
- Large product yields
- Made to excrete product into the medium to ease the recovery process.
- However not suitable for mammalian protein because of no glycosylation
- Insulin, growth hormone and some other are produce through bacterial system

Yeast

- Widely used in BP industry
- Post translational modifications occurs (to some extent).
- Made to excrete product into the medium to ease the recovery process
- One disadvantage is the active production of proteases that destroys proteins which can be engineered.

Transgenic Animals

- First transgenic animal was a mouse produced in yale 1980.
- 1996 Dolly was cloned
- Several companies are producing recombinant protein from transgenic sheep and goats from their milk.

- This technique has several advantages over other systems while making original transgenic animal is complex.
- Yield are comparable to large bioreactors.
- But still potential risk of animal disease transmission.
- Main drawback is the animal welfare.

Transgenic Plants

- Number of therapeutic medicines are produced through tobacco and potato plants.
- Transgenic plants are much cheaper than animals.
- Product purification is costly in plants.
- Use of raw eating plants to avoid extraction and purification step.
- Concentration of products vary with different environment.
- Cross pollination and cross contamination cannot be avoided even with good manufacturing practices.

Mammalian Cell Expression Systems

- First used for the production of inactivated polio vaccine.
- Nowadays continuous immortalized cells lines are used instead of primary cell lines.
- General method is culturing transfected cells on large scale and then target protein is recovered.
- Practicing good manufacturing practices.
- Any small change require change in license product

Good Manufacturing Practice (GMP)

- Adherence to GMP is requirement of the licensing of any drug.
- All the components of manufacturing process must be strictly defined, to make sure that the product is made to the highest levels of safety and efficacy.
- Any change in the system i.e. even to increase the manufacturing product is likely to necessitate a change in the license.

Cell Characterization and Banking

It is necessary to validate the nature and source of all cell lines in the production of BP. Several banks are maintained to ensure the safety and validity of this valuable resource. There would be:

- Two master banks where original stocks are stored
- One or more working banks, where cells are withdrawn for working.

- Banked cells are continuously checked to ensure that they are of right type i.e. genetically stable and not infected or contaminated.
- Age of cells is also monitored.

Product Characterization and Validation

Careful characterization of the product is essential as small change can lead to change in product activity. A single amino acid change can affect activity to 50%. Cells used also effect the nature of product yielded. Changes in the conditions under which cells are cultured can affect the product yield to considerable extent.

LECTURE 97:-

Pharmacogenomics: is the science of how genes affect the way people respond to drugs

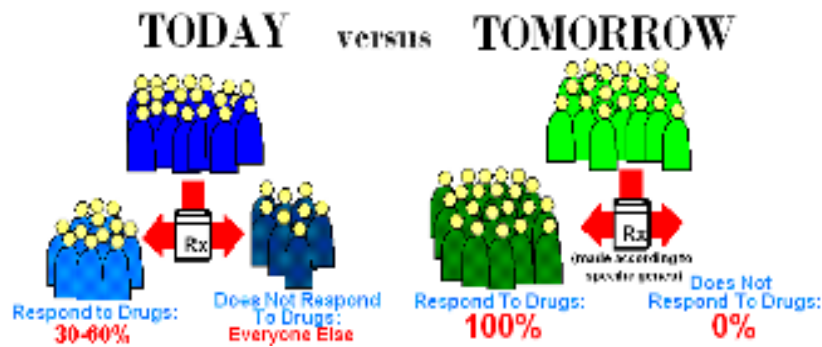
- How genes affect, the way our body processes drugs (pharmacokinetics)
- The interaction of drugs with receptors (pharmacodynamics)
- The treatment efficacy and adverse side effects

Pharmacogenetics

- A subset of 'pharmacogenomics'
- The study of how inherited variation affects drug response and metabolism

Why is this a good approach?

- Drugs can be dangerous
- Many people have severe adverse reactions to drugs
- Many people respond to drugs at different doses
- Many drug treatments are horribly unpleasant, painful
- Drugs are expensive (to take and to make)
- Ineffective drugs are a waste of money to take
- Drug development needs to account for response variability
- Genetics provide a prior information
- Genetics don't change (except in cancer)
- Genetics can point to the cause not just the symptom



Early Studies

- Fast increase in awareness of the interaction of drug and drug response
- Many family studies, twin studies and ‘top-down’ genetic studies
- Very laborious experimental work through the study enzyme actions and clinical observation
- PCR sped things up a bit

CYP2D6

- 1975 Smith and colleagues ingest a drug they are testing
- He had a bad reaction but his colleagues did not
- Family studies revealed genetic inheritance
- Enzyme discovered and characterized
- Enzyme cDNA sequenced and variants found (1990)
- This family of gene important for many drugs

The Technology: Genotyping

- Uses a microarray to measure a limited predefined set of SNPs
- Very high throughput (fast)
- Very inexpensive (cheap)
- Excellent coverage of common variation (up to 5,000,000 SNPs)
- Relies on Linkage Disequilibrium

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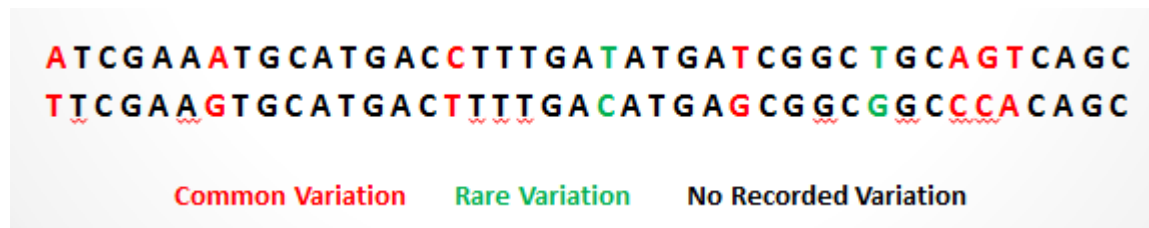
Common Variation

Rare Variation

No Recorded Variation

The Technology: Deep Sequencing

- Captures every base pair in the genome (3,000,000,000)
- (Currently) low throughput (slow)
- (Currently) Very expensive (> 10k)
- Captures common, rare, and personal variation
- New and hard to analyze



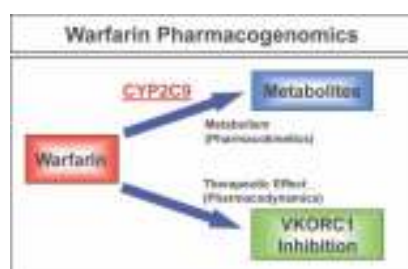
Utility of Drugs

The utility of pharmacogenetics:

- Determining appropriate dosing
- Avoiding unnecessary toxic treatments
- Ensuring maximal efficacy
- Reducing adverse side effects
- Developing or choosing novel treatments
- Can also explain variable response to illicit drugs

Warfarin: A dosage story

- Most widely used anticoagulant in the world
- A “blood thinner”
- Prescribed doses vary widely (1-40mg / daily)
- Therapeutic index is very low
- High risk of bleeding early in treatment
- Two genes involved in metabolism: CYP2C9 and VKORC1



Plavix: A story about effectiveness

- Anti-clotting drug
- Prescribed for coronary artery disease and those who have suffered a heart attack or stroke or have a stent
- A “pro-drug”
- Converted to active form in the liver by CYP2C19

Pegasys: A toxic treatment story

- Pegylated Interferon α -2a
- Interferons are proteins made in response to virus
- Treatment for Hepatitis B and C Virus
- Highly toxic treatment
- Highly variable response, especially in African Americans
- Very expensive

Personalized Medicine

There is an emerging goal among ‘translational scientists’ to make medical practice more personalized. Pharmacogenetics is an important step towards that goal. Personalized medicine is an emerging practice of medicine that uses an individual's genetic profile to guide decisions made in regard to the prevention, diagnosis, and treatment of disease. Knowledge of a patient's genetic profile can help doctors select the proper medication or therapy and administer it using the proper dose or regimen.

Concerns with this Approach

- How reliable are the tests?
- Are health care providers prepared to use this information?
- Will Insurance companies pay for the tests?
- Will tailor made medicine lead to discrimination?
- Will ethnic biases in science bias treatment developments?
- Will this affect people's privacy?

LECTURE 98:-

PRINCIPLES OF DRUG DELIVERY

Drug Delivery : The appropriate administration of drugs through various routes in the body for the purpose of improving health. It is highly interdisciplinary. It is not a young field. It has recently evolved to take into consideration

ORAL ADMINISTRATION

Advantages

- Patient: Convenience, not invasive, higher compliance
- Manufacture: well established processes, available infrastructure

Disadvantages

- Unconscious patients cannot take dose
- Low solubility
- Low permeability
- Degradation by GI enzymes or flora
- First pass metabolism
- Food interactions
- Irregular absorption
- Traditional oral delivery systems
- Tablets
- Capsules
- Soft gelatin capsules
- Suspensions
- Elixirs

BUCCAL/SUBLINGUAL

Advantages

- By-pass First pass metabolism
- Rapid absorption
- Low enzymatic activity

Disadvantages

- Discomfort during dissolution
- Probability of swallowing- lost of effect
- Small doses

RECTAL

Advantages

- By-pass first pass metabolism
- Useful for children

Disadvantages

- Absorption depends on disease state
- Degradation by bacterial flora
- Uncomfortable

INTRAVENOUS (IV)

Advantages

- Drug 100% bioavailable
- Rapid response
- Total control of blood concentration
- Maximize incorporation of degradable drugs

Disadvantages

- Invasive
- Trained personnel
- Possible toxicity due to incorrect dosing
- Sterility

SUBCUTANEOUS

Advantages

- Patient self-administration
- Slow, complete absorption

Disadvantages

- Invasive
- Irritation, inflammation
- Maximum dose volume - 2mL

INTRAMUSCULAR

Advantages

- Patient can administer the drug himself
- Larger volume than subcutaneous
- By-pass first pass metabolism

Disadvantages

- Invasive – patient discomfort
- Irritation, inflammation
- May require some training

INHALERS

Advantages

- Gases are rapidly absorbed
- Easy to use

Disadvantages

- Solids and liquids can be absorbed if size is below 0.5um

Factors Influencing the Selection of the Delivery Route

- pH
- pKa - ionization
- Temperature
- Concentration
- Crystallinity

- Particle size
- State of hydration

Factors Influencing Bioavailability

- Delivery route
- The site of measurement
- Type of animal employed
- Physiological state of the animal/human

LECTURE 99:- Synthetic Pharmaceuticals

Drug & Medicine

Drug is a chemical substance that interacts with a living system and produces a biological response. **Drugs** are considered to be such things as stimulants, narcotics, and hallucinogens, and often are used in the medical field to treat or cure disease. **Medicine**, however, is a term for a drug when that drug is used to cure, mitigate, treat, or prevent a disease.

What criteria MUST new drugs meet?

Drugs must address a new need or provide a significant “added benefit” over an existing medicine. Drugs must also meet these criteria:

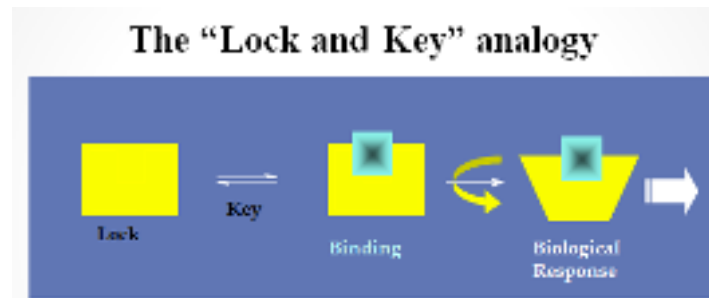
- Must be safe
- Effective
- Of high quality
- Cost effective

Major Therapeutic Targets

Infectious disease – anti-infectives: Anti-bacterial, anti-viral, anti-parasitic drugs

Metabolic disease: cancer, cardiovascular, diabetes, inflammation, high blood pressure, neurological disease, pain

Other aspects of health care: Hormonal treatments, contraception, vaccines, immunosuppressant, anaesthetics, nutraceuticals,



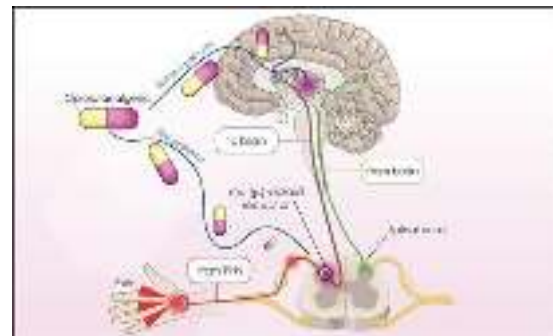
Here the KEY is the natural substrate. Binding of the KEY to the LOCK (an enzyme or a receptor) then causes a response – a shape change in the protein/receptor

ANALGESICS

Analgesic drugs act in various ways on the peripheral and central nervous systems. They are distinct from anesthetics, which reversibly eliminate sensation.



Mechanism of action



Paracetamol and NSAIDs

The exact mechanism of action of paracetamol/acetaminophen is uncertain but appears to act centrally in the brain rather than peripherally in nerve endings. Aspirin and the other non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenases, leading to a decrease in prostaglandin production. In contrast to paracetamol and the opioids, this reduces not only pain but inflammation as well.

OPIOIDS:

Morphine, the archetypal opioid, all exert a similar influence on the cerebral opioid receptor system. Opioids, while very effective analgesics, may have some unpleasant side-effects. Patients starting morphine may experience nausea and vomiting, itching, constipation etc. When used appropriately, opioids and similar narcotic analgesics are otherwise safe and effective, however risks such as addiction and the body's becoming used to the drug (tolerance) can occur. The effect of tolerance means that frequent use of the drug may result in its diminished effect so, when safe to do so, the dosage may need to be increased to maintain effectiveness

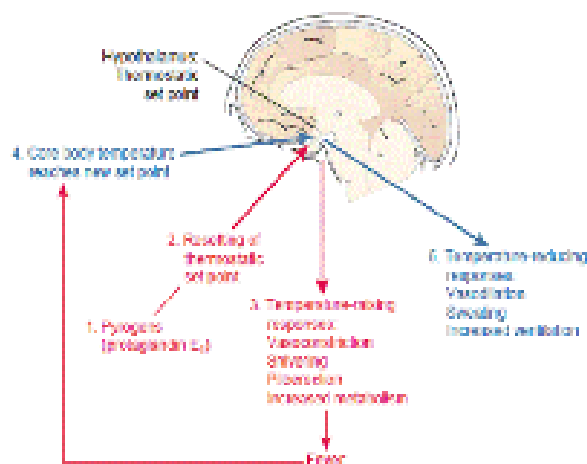
Anti-inflammatory

- **Anti-inflammatory** refers to the property of a substance or treatment that reduces inflammation or swelling.
- COX-2 inhibitors
- Prostaglandins inhibitors

Inflammation

Cardinal Signs	Physiological rationale
1. Rubor (Redness)	Increased Blood flow
2. Tumor (Swelling)	Exudation of fluid
3. Calor (Heat)	Increased Blood flow, Exudation of fluid, Release of inflammatory mediators
4. Dolor (Pain)	Stretching of pain receptors and nerves by inflammatory exudates, chemical mediators
5. Functio laesa (Loss of function)	Pain, Disruption of tissue structure, Fibroplasia and metaplasia

Antipyretic: Antipyretics cause the hypothalamus to override an interleukin-induced increase in temperature.



Antiemetic: Antiemetic is a drug that is effective against vomiting and nausea. Block serotonin receptors in the central nervous system and gastrointestinal tract

Antihistamine: Antagonists of the histamine H₁-receptor are used to treat allergic reactions. Histamine produces increased vascular permeability, causing fluid to escape from capillaries into tissues, which leads to the classic symptoms of an allergic reaction — a runny nose and watery eyes.

Steroid (Life Saving Drug): Steroids reduce the production of chemicals that cause inflammation. This helps keep tissue damage as low as possible. Steroids also reduce the activity of the immune system by affecting the way white blood cells work. Given when,

- Body defense is poor
- Extensive tissue damage

ANESTHETICS: An **anesthetic** is a drug that causes anesthesia, which is a reversible loss of sensation.

1. General anesthetics

2. Local anesthetics

Antibiotics: Medications used to treat bacterial infections

1. Sulfonamides

2. Penicillins

3. Cephalosporins

4. Tetracyclines

5. Aminoglycosides

6. Quinolones

7. Macrolides

How Antibiotics Work

- Block protein formation (Macrolides, Tetracyclines, Aminoglycosides)
- Inhibit cell wall formation (Pencilline, Cephlosporins)
- Interfere with DNA formation (Nalidixic acid (synthetic quinilone) , Quinilone)
- Prevent folic acid synthesis (Sulfonamides)

Others

Diuretics

Anti- depressants

Contraceptives

Anti-hypertensive

Athletic steroids

Anti-coagulants

100. DRUG ABUSE

What are drugs and What is their normal use?

A chemical substance that is used in treatment, cure, prevention, or diagnosis of disease or used to otherwise enhance physical or mental well-being. They altering biochemical and physiological process of tissue or organism.

Street Drug: Drug that are taken for non medical reasons (usually for mind altering effects)

Can lead to

- Physical damage
- Mental damage
- Dependence and addiction

Include alcohol, heroin, methamphetamine, crack, cocaine and marijuana (Cannabis)

Drug Abuse: A form of substance-related disorder. Patterned use of a drug in which the user consumes the substance in amounts or with methods which are harmful to themselves or others. Drugs most often associated with this term include: Alcohol, substituted amphetamines, barbiturates, benzodiazepines, cocaine, methaqualone, and opioids.

Drug Addiction: Chronic, often relapsing brain disease that causes compulsive drug seeking and use, despite harmful consequences to the addicted individual and to those around drug addicts.

Difference between drug abuse and addiction

- **Drug abuse** refers to using drugs even though it has become a problem in life.
- **Drug addiction** refers to the substance, taken in higher amounts or taken over a longer period of time than the user intended.

Cause of Substance Abuse: Not clear, with theories including:

- A genetic disposition
- Learned from others
- A habit which if addiction develops, manifests as a chronic debilitating disease

Genetics: Factors that make it harder to become addicted also may be genetic. Example: an individual may feel sick from a drug that makes other people feel good. There is no single addiction gene but many interacting genes. Social and environmental factor contribute to risk of addiction

Other Factors

1. **Gender:** Men are twice as likely to have problems with drugs

2. **DRUG:** Cause temporary, pleasurable feelings, Dependent

3. **Mental condition:** Anxiety, depression and other mental conditions

Individual factors	Environmental factors	Constitutional factors
Personality	Socioeconomic status	Biological/genetic predisposition to use/misuse drugs
Gender	Poverty	
Anxiety	Delinquency	
Stress	Family background/disturbance	
Power needs	Peer pressure	
Age	Ideology/religion	
Intelligence	Educational opportunities	
Psychological health	Educational disturbance	
Life events	Truancy	
Predisposition to take risks	Drug availability	
Hedonism	Drug price	
Self-destructiveness	Unemployment/job opportunities	
Curiosity	Anomie (lack of social/moral standards)	
	Alienation (rebelliousness)	
	Tradition	
	Legal arrangements	
	Historical factors	

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Conclusion: Just because you are prone to addiction doesn't mean you're going to become addicted. It just means you've got to be careful. There are many ways that genes could cause one person to be more vulnerable to addiction than another.

101: Blood Transfusion

Blood transfusion is defined as the process of receiving blood products into one's circulation intravenously. This is usually done as a life saving maneuver to replace blood cells or blood products lost through severe bleeding, during surgery when severe blood loss occurs or to increase the blood count in an anemic patient. Blood transfusions involve the use of whole blood, red blood cells, white blood cells, plasma, clotting factors and platelets.

History of Transfusion

Blood transfused in humans since mid- 1600's

- 1828 – First successful transfusion
- 1900 – Landsteiner described ABO group
- 1916 – First use of blood storage
- 1939 – Levine described the Rh factor

Purpose

- To replace blood lost during surgery or a serious injury.
- To restore oxygen-carrying capacity of the blood.
- To provide plasma factors to prevent or treat bleeding.
- Done if patient's body is not capable of making blood properly because of an illness.

Blood Types and Compatibility

- Each person has one of the following blood types:
- A, B, AB, or O.
- can be given to anyone but can only receive O.
- AB can receive any type but can only be given to AB.
- Also, every persons blood is either Rh-positive or Rh-negative.

Tranfusion

A Better way to Say "Get Well Soon"

Donate Blood Save Life

Type	You Can Give Blood To	You Can Receive Blood From
A+	A+, AB+	A+, A-, O+, O-
O+	O+, A+, B+, AB+	O+, O-
B+	B+, AB+	B+, B-, O+, O-
AB+	AB+	Everyone
A-	A+, A-, AB+, AB-	A-, O-
O-	Everyone	O-
B-	B+, B-, AB+, AB-	B-, O-
AB-	AB+, AB-	AB-, A-, B-, O-

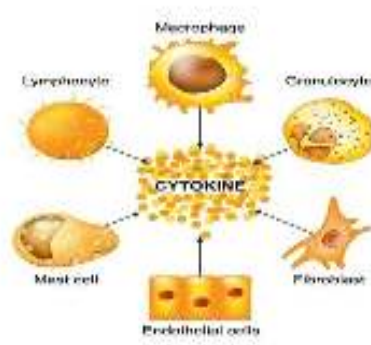
Transfusion

- The blood used in a transfusion must be compatible with the patients blood type.
- Type O blood is called the universal donor
- People with type AB blood are called universal recipients
- People with Rh-positive blood can get Rh- positive or Rh-negative blood. But people with Rh-negative blood should get only Rh- negative blood.

Blood Banks : Blood banks collect, test, and store blood.

Autologous transfusion - If surgery is scheduled months in advance, patients may be able to donate their own blood and have it stored.

Cytokines: Derived from the Greek word “cyto” means cell and “kinos” means moment.



Cytokines are low molecular weight regulatory proteins or glycoprotein's (8~80 KD) secreted by white blood cells and various other cells in the body in response to a number of stimuli

General properties of cytokines

2. Cytokines are produced by a variety of cell types
3. Many cell types can produce more than one cytokine
4. Many cytokines play a regulatory role in processes of immunity and inflammation
5. Most cytokines are pleiotropic
6. Most cytokines are inducible and secreted by their producer cells
7. Many cytokines exhibit redundancy

Classification of cytokines

Table The major proteins/protein families that constitute the cytokine group of regulatory molecules

The interleukins (IL-1 to IL-33)
The interferons (IFN- α , - β , - γ , - ω)
CSFs (C-CSF, M-CSF, GM-CSF)
TNFs (TNF- α , - β)
The neurotrophins (NGF, brain-derived neurotrophic factor (BDNF), NT-3, NT-4/5)
Uiliary neurotrophic factor (UNIT)
Glial cell derived neurotrophic factor (GDNF)
EGF
EPD
Fibroblast growth factor (FGF)
Leukemia inhibitory factor (LIF)
Macrophage inflammatory proteins (MIP-1 α , -1 β , -2)
PDGF
Transforming growth factors (TGF- α , - β)
TPD

*M-CSF: macrophage-colony-stimulating factor; NT: neurotrophin

Interferon

Interferon's (IFNs) are proteins made and released by host cells in response to the presence of pathogens such as viruses, bacteria, parasites or tumor cells. In a typical scenario, a virus-infected cell will release interferon causing nearby cells to ramp up their anti-viral defenses.

There are three basic families of interferon

1. IFN- α –consist of 166 to 172 A.A having 19-20 kDa mass
2. IFN- β - consist of 166 A.A having 20 kDa mass
3. IFN- γ - consist of 143 A.A having 17 kDa mass

Important Function of INFs

- Induction of cellular resistance to viral attack;
- Regulation of most aspects of immune function;
- Regulation of growth and differentiation of many cell types;
- Sustenance of early phases of pregnancy in some animal species.

Clearance of Viral Infected Cell

Exposure of cells to interferon normally results in the induction of a protein kinase termed eIF-2 α protein kinase. The enzyme, which is synthesized in a catalytically inactive form, is activated by exposure to dsRNA. The activated kinase then phosphorylates its substrate, i.e. eIF2 α , which is the smallest subunit of initiation factor 2 (eIF2). This, in turn, blocks construction of the smaller ribosomal subunit, thereby preventing translation of all viral (and cellular) mRNA

Uses of interferon

- iNF- α 2a and 2b are effective against leukemia and malignant tumor of bone marrow
- iNF- α 2b is approved and effective against hepatitis B and C
- iNF- α 3n and rh-iNF are approved for sexually transmitted genital warts caused by papilloma virus
- iNF β is effective against the chronic disease of NS
- iNF γ effective to treat lieshmania disease caused by parasitic protozoa stay inside the macrophages

Interleukins

Interleukins represent large family of cytokines, with at least 33 different constituent members (IL-1 to IL-33). Only two interleukin-based products have gained approval for general medical use (Proleukin an IL-2 and Neumega an IL-11)

Many cell types are capable of producing a whole range of interleukins.

- IL-2, also known as T-cell growth factor.
- Human IL-2 is a single-chain polypeptide containing 133 amino acids.
- The mature molecule displays a molecular mass ranging from 15 to 20 kDa.
- Initially expressed in E. coli.
- Large-scale IL-2 production was made possible by recombinant DNA technologies.

Therapeutic Aspects of Tumor Necrosis Factor

Many tumors are not susceptible to destruction mediated by TNF (indeed, some tumors produce TNF as an autocrine growth factor). Tumor cell necrosis is not TNF's major biological activity. Severe side effects usually accompany systemic administration of therapeutically relevant doses of this cytokine

Some diseases in which TNF is known to mediate many of the symptoms

Disease	Symptoms Induced by TNF
Cancer	Cachexia Stimulation of growth of some tumours
Septic shock	Vascular leakage Tissue necrosis Hypotension Activates blood clotting
Rheumatoid arthritis	Tissue inflammation Possible role in destruction of joints
MS	Inflammation
Diabetes	Death of pancreatic islet cells induces insulin resistance

Typing and Cross Matching

Before any blood can be given to a patient, it must be determined that the blood of the donor is compatible with the patient. The laboratory examination to determine a person's blood group and Rh factor is called Blood Typing. The process of determining compatibility between blood specimens is Cross matching. RBCs from the donor blood are mixed with serum from the recipient, a reagent (Coombs's serum) is added, and the mixture is examined for visible agglutination. If no antibodies to the donated RBCs are present in the recipient's serum, agglutination does not occur and the risk of transfusion reaction is small.

Blood products

- Components of the blood which are collected from a donor for use in blood transfusion.
- Packed red Blood Cells (*PRBCs*)
- Fresh Frozen Plasma (FFP)
- Platelets

Side Effects

Hemolytic reactions: the patient's own blood destroys the transfused blood. This is due to a human error. Careful patient identification steps are followed to make sure the correct blood is given.

- Bacterial infection (highest with platelet transfusions)
- Itching, skin rash, fever, or feeling cold
- More serious side effects such as trouble breathing are very rare
- HIV (AIDS)
- Hepatitis C
- Hepatitis B

FLOW CYTOMETRY

Flow cytometry is a technique of quantitative single cell analysis. This technique was first described by Wallace Coulter in the 1950s. The flow cytometer was developed in the 1970's and applied to automated cell counting. Flow cytometer count, examine and sort cells based on their optical properties (Scattering and fluorescence). The present "state-of-the-art" flow cytometers are capable of analyzing upto 13 parameters (forward scatter, side scatter, 11 colors of immunofluorescence).

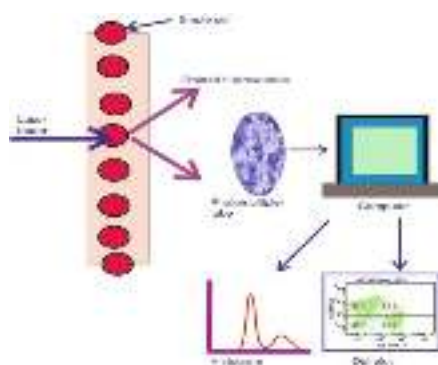
Components of Flow Cytometer

1. Lasers
2. Dichroic mirrors
3. Filters

4. Detectors

Basic Principle of Flow Cytometry: Prepared single cell or particle suspension is necessary for flow cytometric analysis. The suspension of cells or particles is aspirated into a channel surrounded by a narrow fluid system. They pass one at a time through a focused laser beam. The light is either scattered or absorbed when it strikes a cell.

Principle: Light scattering is dependent on the internal structure of the cell and its size and shape. Absorbed light of the appropriate wavelength may be re-emitted as fluorescence. (The cell may have a naturally fluorescent substance or one or more fluorochromelabelled antibodies are attached to surface or internal cell structures). Light and/or fluorescence scatter signals are detected by a series of photodiodes and amplified. Optical filters are essential to block unwanted light and permit light of the desired wavelength to reach the photodetector. Fluorescein isothiocyanate (FITS), Texas red and phycoerythrin (PE) are the most common fluorescent dyes used in the biomedical sciences. Large number of cells are analyzed in a short period of time (>1,000/sec).



Working Process



Working of Flow Cytometer

A flow cytometer is composed of three main systems:

Fluidics – Transport cells in a stream to the laser beam for interrogation.

Optics – Consist of lasers to illuminate the cells in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors.

Electronics – Converts the detected light signals into electrical signals that can be processed by computer.

Fluidics System

Flow cytometers use the principle of hydrodynamic focusing for presenting cells one at a time to a light source. The fluidics system consist of a central channel through which the sample is injected, enclosed by an outer sheath that contains faster flowing fluid.

Optics

Light scattering or fluorescence emission provides information about the cell's properties. Light that is scattered by an object is detected by different detectors. One detector is placed in line with the beam to measure the forward scatter (FSC) from the objects. Detectors perpendicular to the beam measure side scatter (SSC) and fluorescence.

Optical Detectors

Scattered and emitted light from cells are converted to electrical pulses by optical detectors. Once a cell or particle passes through the laser light, the scattered and fluorescence signals are diverted to the detectors. Detectors are either silicon photodiodes or photomultiplier tubes (PMTs). The photodiode is less sensitive to light signals than the PMTs and thus is used to detect the stronger FSC. PMTs are used to detect the weaker signals generated by SSC and fluorescence.

Types of Filter

There are three major type of filters:

- **Long pass Filter:** Transmits wavelength of light equal to or greater than the spectral band of the filter.
- **Short pass Filter:** Transmits wavelength of light equal to or shorter than the spectral band of the filter.
- **Bandpass Filter:** Transmits wavelength of light within a specific range of wavelengths.

Electronics (Signal Processing)

Scattered and emitted light data can be converted to electrical pulses by optical detectors. Flow cytometry data may be represented as histograms or dot plots.

Clinical Applications of Flow Cytometry

- Diagnosis of Hematologic Malignancies
- Detection of Minimal Residual Disease
- Lymphocyte Subset Enumeration (HIV)
- Efficacy of Cancer Chemotherapy
- Reticulocyte Enumeration
- Cell Function Analysis
- Application in Organ Transplantation

Transcriptome: The transcriptome is the complete set of transcripts in a cell and their quantity, for a specific developmental stage or physiological condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, and also for understanding development and disease. 

Transcriptomics: Transcriptomics, the study of RNA in any of its forms. The transcriptome is the set of all RNA molecules, including mRNA, rRNA, tRNA, and other non-coding RNA produced in one or a population of cells.

Transcriptomics scope: The term can be applied to the total set of transcripts in a given organism, or to the specific subset of transcripts present in a particular cell type. Unlike the genome, which is roughly fixed for a given cell line (excluding mutations), the transcriptome can vary with external environmental conditions.

Transcriptomics scope: Because it includes all mRNA transcripts in the cell, the transcriptome reflects the genes that are being actively expressed at any given time, with the exception of mRNA degradation phenomena such as transcriptional attenuation (Premature termination of transcription). The study of transcriptomics, also referred to as expression profiling, examines the expression level of mRNAs in a given cell population, often using high-throughput techniques based on DNA microarray technology.

Transcriptomics Aims

1. To catalogue all species of transcripts, including mRNAs, noncoding RNAs and small RNAs.
2. To determine the transcriptional structure of genes, in terms of their start sites, 5' and 3' ends, splicing patterns and other post-transcriptional modifications.
3. To quantify the changing expression levels of each transcript during development and under different conditions.

TECHNOLOGIES

Hybridization-based approaches

- Fluorescently labelled cDNA with custom-made microarrays
- Commercial high density oligo microarrays

Sequence-based approaches

- o Sanger sequencing of cDNA or EST libraries
- o Serial analysis of gene expression (SAGE)
- o Cap analysis of gene expression (CAGE)
- o Massively parallel signature sequencing (MPSS)

Applications

- One way to directly or indirectly identify biomarkers is with the use of transcriptomics.
- This technique enables the analysis of the entire set of RNA transcripts in an organism.
- The transcriptome consists of many different RNA species that are categorized into messenger RNAs (mRNA) and non-coding RNAs

PROTEOMICS

The terms proteome and proteomics were coined by Mark Wilkins and colleagues in the early 1990's

Proteome : is the complement protein found in a single cell in a particular environment/ is complete collection of proteins encoded by genome of an organism.

Proteomics : is the study of composition, structure, function and interaction of the proteins directing the activities of each living cell

Bacterial transcriptome analysis could lead to the identification of novel antigens

TYPES OF PROTEOMICS

Interaction proteomics: protein-protein association

Expression proteomics: protein quantification

LIFE CYCLE OF PROTEIN

The level of any protein in the cell at any given time is controlled by

- o Rate of transcription of the gene
- o The efficiency of translation of m RNA into protein
- o The rate of degradation of protein in the cell

Protein structure

Primary structure: is sequence of specific amino acid

Secondary structure: the primary polypeptide chain gets properly folded In the form of alpha-helix and β -pleated sheets

Tertiary structure: secondary structure interact with each other chemically to form the 3 dimensional shape of the proteins.

Quaternary structure: interaction between different polypeptide unit helix, beta pleated sheet, random coils and turns

Domains: discrete portions of the proteins that fold independently from the rest of protein and they have their own function and serve as one of the building blocks of that proteins.

Motif: domains contain a region of conserved pattern of amino acids or a conserved combinations of the structural elements formed by folding of near by amino acid sequences.

Determining the protein structure/ polypeptide sequence by:

- X-ray crystallography
- Nuclear magnetic resonance
- Computer based protein predicting programs

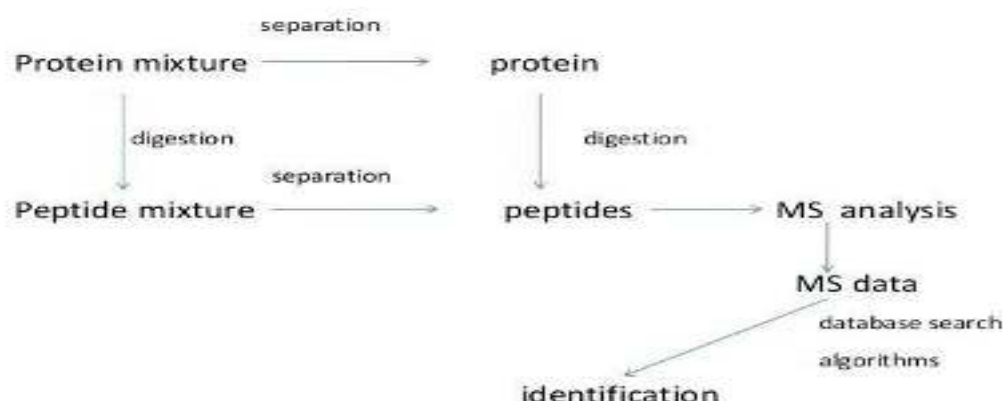
POST TRANSLATIONAL MODIFICATIONS

Glycosylation: structural components of cell surface helps in binding by the receptors and eliciting immune response. eg. ABO blood group antigen, Human IgG

Phosphorylation: Play a role in the regulation of many cell processes such as growth and cell cycle control.

Sulfation: Plays role in strengthening protein-protein interactions

Tools of proteomics



Applications of Proteomics

1. Mining-Identification of all of the proteins in a sample

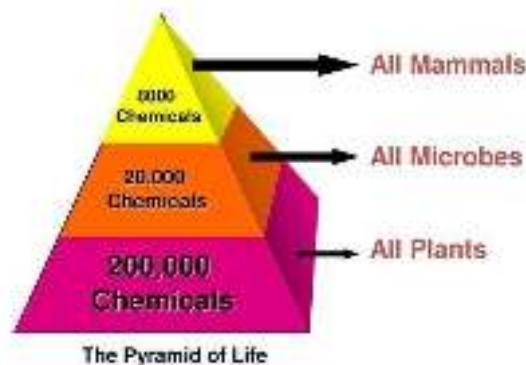
2. **Protein expression profiling**-Identification of proteins in a sample as a function of particular state of a cell i.e., two states of a particular systems are compared
3. **Protein network mapping**-To determine protein interaction with each other in a living system
4. **Mapping of protein modifications**-Task to identify how and where proteins are modified post translationally

METABOLOMICS

Metabolomes are group of small molecules- Metabolites o Chemical molecules o Essential in metabolic processes o Example : proteins , lipids

Metabolome: The complete collection of small molecule chemicals found within a biological sample. The biological sample can be a cell, a cellular organelle, an organ, a tissue, a tissue extract, a biofluid or an entire organism

Different Metabolomes



Metabolites

- Intermediates and products of metabolism
- MW < 1500 Da
- Examples- antibiotics, pigments, carbohydrates, fatty acids and amino acids
- Primary and secondary metabolites

Metabolomics

- Newly emerging field of 'omics' research
- Field of Comprehensive and simultaneous systematic determination of metabolites
- It includes, study of levels in the metabolome, Types
- Deals with function, Structural changes, Effects
- Also known as metabonomics

One of computational science among omics. It involves in quantitative measurement of the dynamic multi- parametric metabolic response of living systems. Deals with, systematic identification and quantitation of all/ biologically relevant small molecules in a given compartment, cell, tissue or body fluid. It represents the functional end-point of physiological and pathophysiological processes depicting both genetic predisposition and environmental influences like nutrition, exercise or medication

AIMS

Four main points in Analysis of metabolomics Data:

- Efficient and unbiased
- Separation of analytes
- Detection
- Identification and quantification

Separation techniques

- Gas chromatography (GC)
- Capillary electrophoresis (CE)
- High performance liquid chromatography (HPLC)
- Ultra performance liquid chromatography (UPLC)

Combination of techniques:

- GC-MS
- HPLC-MS

DETECTION TECHNIQUES

- Nuclear magnetic resonance
- Mass spectrometry

Importance of metabolites

- >95% of all diagnostic clinical assays test for small molecules
- 89% of all known drugs are small molecules
- 50% of all drugs are derived from pre-existing metabolites
- 30% of identified genetic disorders involve diseases of small molecule metabolism
- Small molecules serve as cofactors and signaling molecules to 1000's of proteins

APPLICATIONS

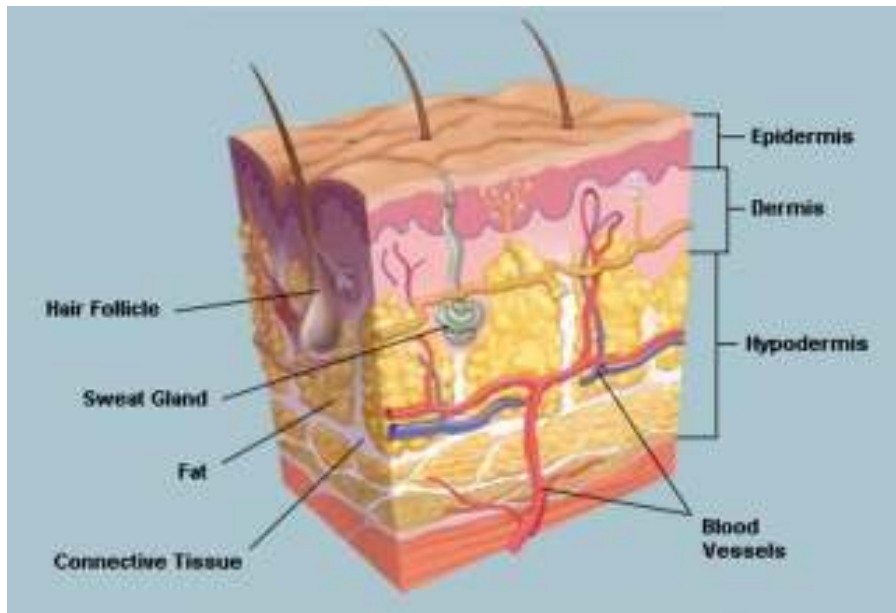
1. Toxicology Testing
2. Clinical Trial Testing
3. Fermentation Monitoring
4. Food & Beverage Tests
5. Nutraceutical Analysis
6. Drug Phenotyping

7. Water Quality Testing

8. Petrochemical Analysis

Bioartificial Skin : The term artificial skin is used to describe any material used to replace (permanently or temporarily) or to mimic the dermal and epidermal layers of the skin. The primary current application of artificial skin is for the treatment of skin loss or damage on burn patients. Alternatively however, artificial skin is now being used in some places to treat patients with skin diseases, such as diabetic foot ulcers etc.

Anatomical Overview of Skin : Human skin is comprised of two primary layers, the dermis and the epidermis. A diagram of a typical section of human skin is shown here. The epidermis contains no blood vessels meaning transport of nutrients to the epidermis occurs from the dermis below:



Historical Overview

- 1871, England: Pollock proposes skin grafts for burn treatment
- 1998, United States: First tissue engineered skin is approved by the FDA
- 2001, United States: Dermagraft, Orcel, Composite Cultured Skin Graft are FDA approved
- 2002, United States: Integra is FDA approved for treatment of severe burns

How Artificial skin is Made

Skin is usually donated by other donors. Fibroblasts are removed from the donated skin and are frozen until they are needed. The fibroblasts are placed on a polymeric mesh scaffolding,

gather oxygen, and grow new cells. The cells are then transferred to a culture system. After 4 weeks the polymer mesh dissolves and leaves behind a new layer of dermal skin. When the growth cycle is completed, they add more nutrients. Keratinocytes are added to the collagen and are exposed to air to form epidermal layers. The skin is now completed and is stored in sterile containers until ready to use

How It Works

Artificial skin is secured over wound during surgery. The skin remains in place for several weeks and allows new tissue to grow in to bottom matrix layer. Top layer provides protection from infection and dehydration

Companies: Bioartificial skin is produced by many companies but the initial ones are

1. Integra
2. Advance tissue sciences (ATS)
3. Organogenesis

These companies grow their skin and provide it to others. Ultimate source of skin is foreskin after infant circumcision. These infantile cells have the ability to divide and have long life as compared to adult cells

Areas of Applications

- Artificial Skins are primarily used for the Treatment of Skin loss or damage on burn Patients.
- Alternative Areas of Application of Artificial Skins includes
 - Treatment of patients with skin diseases, such as diabetic foot ulcers, and severe scarring.
 - Plastics and Cosmetic Surgery.

Advantages

- Chances of survival for burn patients
- Artificial skin seals the wound preventing fluid loss and bacteria from entering through the wound.
- The fear of Stigmatization of the Patient is eliminated

Disadvantages

- Risks of Infection and Rejection by the Patients
- Loss of Sensitivity
- Cut of Blood Supply
- Complication could arise due to Skin Adhesion and/or fluid buildup between the wounded site and the transplanted skin
- Artificial Skins are very expensive

Current Research

Though artificial skin has aided significantly in skin regeneration, there remain several areas for improvement. Ideally, this would allow in vitro replication of a patient's own genetically modified skin cells. These cells could then be put into the artificial matrix for bacteria-free growth. Another current trend in Artificial is the creation of Electronic Skin. Scientist are working towards the Incorporation of flexible pressure transducers and Bio-receptors to the Artificial Skin, these will give a sense of Touch to the Patients.

Conclusion: The ultimate goals of current artificial skin technologies are to provide protection from infection, dehydration, and protein loss after severe skin loss or damage.

109. BIOARTIFICIAL KIDNEY

Kidneys: Kidneys are the main excretory organs of the body. Located near the back of the abdomen behind the intestines. The kidneys are two reddish-brown bean shaped organs 10cm long and 6cm wide.

Main Functions of Kidney

- Ion Balance - Especially Na^+ and K^+
- Osmotic Balance - Determine volume of urine produced
- Blood Pressure - Controls blood volume and ECF volume
- pH Balance - Retains or excretes H^+ or HCO_3^-
- Excretion - of nitrogenous wastes & other hydrophilic toxins
- Hormone Production-Renin, Erythropoietin

Artificial Kidney (Hemodialyzers): Artificial kidneys or hemodialyzers are used in extracorporeal renal-therapies for removal of uremic solutes and excess plasma water from the blood of patients with kidney failure. **History of Artificial Kidney**

- The first scientific description of hemodialysis principle was published by Graham in 1854

- The first prototype hemodialyzer was developed by Abel, Rowntree and Tunner in 1913
- The first hemodialyzer used on human was reported by Hass in 1923.
- Kolf developed the rotating drum artificial kidney in 1945 and is succeeded in treating ARF for the first time and it is modified in 1956
- In 1960, a plate and frame hemodialyzer called Killi dialyzer was developed by Killi.
- In 1964, the hollow-fiber hemodialyzers called the capillary kidney was first proposed by Stewart, Creny and Mahon.
- Hollow-fiber type hemodialyzers are the most widely used

How does Artificial Kidney works?

Artificial kidney or hemodialyzer works on the principle of dialysis which is the diffusion of small solute molecules through a semi permeable membrane. Blood is removed from the body and pumped by a machine outside the body into a dialyzer (artificial kidney). The dialyzer filters metabolic waste products from the blood and then returns the purified blood to the person. The total amount of fluid returned can be adjusted. A person typically undergoes hemodialysis at a dialysis center. Dialysate is the solution used by the dialyzer

HD consists of perfusion of heparinized blood and physiologic salt solution on opposite sides of a semi permeable membrane. Waste products (urea, creatinine,...etc) move from blood into the dialysate by passive diffusion along concentration gradient

Diffusion rate depends on;

1. The difference between solute concentrations in the blood and dialysate
2. Solute characteristics
3. Dialysis filter composition
4. Blood and dialysate flow rate

Dialysis membrane: Dialysis membrane acts as a semi permeable barrier for the removal of uremic solute and excess plasma water from the plasma water. They vary according to their morphology and chemical composition. The morphology determines transport properties of the uremic solute while chemical composition determines the biocompatibility of the molecule.

Classification of Dialysis Membrane: Dialysis membrane can be classified into three based upon their polymeric composition and preparation process. They are:

1. Regenerated cellulosic membrane
2. Modified cellulosic membrane

3. Synthetic membrane

110. BIOARTIFICIAL ORGANS

Artificial Organ: An Artificial organ may be defined as a human made device designed to replace, duplicate or augment, functionally or cosmetically a missing, diseased or otherwise incompetent part of the body, either temporarily or permanently and which requires a non-biologic material interface with the living tissue.

Introduction: A biological device that contains a biological component and a device component that function in a unique association

- **Biologic component:** Isolated cells, cell lines or living tissues.
- **Synthetic component:** biopolymers, plastic or permeability selective membranes among other parts.

Different Aspects of Artificial Organs: Artificial organs can conveniently be classed into four groups:

- Bone/Joint Replacements (e.g. hip, knee, finger, total limb),
- Skin/Soft Tissue Replacements (e.g. skin, breast, muscle),
- Internal Organs (e.g. heart, kidney, blood vessels, liver, pancreas)
- Sensory Organs (e.g. eye, ear).

Manufacturing Processes of Artificial Organs

The various processes involved in the manufacturing of artificial organs involve the following:

- **Tissue Engineering:** This is the use of a combination of cells, engineering and materials methods, and suitable biochemical and physico-chemical factors to improve or replace biological functions.
- **Bioprinting or 3D printing of tissues:** Bioprinting, where living cells are precisely printed in a certain pattern, has great potential and promise for fabricating engineered living organs.
- **Industrially** by using biomaterials which includes metals, ceramics, polymers and composites.
- In the laboratory by using stem cells under **Regenerative Medicine**

Areas of Applications

- Artificial Bone
- Artificial Skin
- Bionics

- Biomedical Engineering
- Cochlear Implant
- Ocular prosthetic
- Organ Anatomy
- Organ Transplant
- Plastic Surgery
- Prosthesis
- Tissue Scaffold

Advantages

1. Artificial organs can replace diseased or damaged organs, thereby, providing the ailing patient with an opportunity to lead a healthy and normal life.
2. Artificial organs can meet the huge demand of healthy donor organs. There is a huge list of patients who are in urgent need of healthy organs but are unable to find a suitable willing donor.
3. A major stumbling block in the form of organ rejection can be solved due to artificial organs. As artificial organs are created by taking the stem cells of the same person and of the same organ, the possibility of rejection has been reduced significantly.
4. With the help of regenerative medicine or artificial organ therapy, burn victims can even have a new skin.

Disadvantages

1. A major concern is the possible presence of the disease in the base tissue which is used to create the organ. Sometimes, even a foreign body tissue is used to regenerate or reconstruct the organ. In such cases, there is a possibility that the tissue is already infected by other diseases.
2. The entire cost of growing and transplanting an artificial organ is prohibitive, and thus, limit the scope of its application to the general public.
3. There are high chances of organ failure, and the body may even take some time to adapt to the new organ. How the body reacts to the new organ may vary from person to person.
4. If there is a problem with the functioning of the organ, you might need to go for another transplant

111. BIOARTIFICIAL PANCREAS

Normal Functioning Pancreas: The pancreas is an organ located in the abdomen. It plays an essential role in converting the food we eat into fuel for the body's cells. The pancreas has two main functions: an exocrine function that helps in digestion and an endocrine function that regulates blood sugar.

Why the need for an Artificial pancreas?

- Pancreas fails to create proper amounts of insulin to counter blood glucose levels
- Patients must frequently screen blood glucose by finger sticks
- Self-injections or machine injections of insulin
- Difficult to determine correct amount of insulin based on meals, exercise, stress and changing schedules
- Diabetes maintenance is tedious and expensive
- The FDA has been cooperating with diabetes patients, care providers, medical device manufactures, and researchers to progress the advancement of an artificial pancreas

Bioartificial Pancreas: Bioartificial pancreas has the ability in preventing or reversing complications associated with the disease. Construct is based on encapsulation of islet cells with a semipermeable membrane so that cells can be protected from host immune system. Encapsulation of islets eliminates the requirement of immunosuppressive drugs

Requirements for Bioartificial Pancreas

1. A steady supply of islets cells
2. Islets cell viability must be maintained with appropriate oxygen supply and nutrients
3. Islets must be protected by immune system
4. Practical means of implantation must exist
5. Hydrogel hybrid-type organs or pancreas.
6. In this system islets are encapsulated inside a semipermeable membrane, which secrete specific compounds in response to body fluids.

Two methods used to obtain such systems are

- Microencapsulation
- Special large containers.

Encapsulation: First attempt is based on additional covering of alginate matrix by poly vinyl layer crosslinked with glutaraldehyde. Second approach is additional covering of alginate matrix by poly (methyl methacrylate) membrane. By selecting appropriate condition of

encapsulation low molecular weight compounds and low molecular weight proteins can be allowed to pass through the semipermeable membrane.

Design objectives:

Keep the cells alive

- Material containing islets is biocompatible
- Process for fabrication must not damage islets
- Dimensions permit rapid diffusion of nutrients

Prevent destructive Host response

- Outer surface should be totally bio invisible
- All islets must be completely covered to prevent immune response
- Permeability is controllable

Assure practical surgical implantation

- Islets density must be high
- Device retrievable
- Bio-stable long –term
- Chemically and physically durable

Advantages of Bioartificial Pancreas:

1. No immune suppression required
2. Xenografts (use of animal tissue)
3. Retrievable
4. Replaceable.

Disadvantages

1. To date no product in in market.
2. Cells inside the encapsulation die because of no oxygen availability because of induction of immune response.
3. Dimensions does not allow penetration of oxygen towards the core and cells die.

112. CLONING STRATEGIES

Cloning: 'Cloning' describes many different processes used to produce genetically identical copies of biological matter. The copied material is called the 'clone'. The clone has the same genetic makeup as the original.

Clones:

- 1938 – the first idea of cloning
- 1952 – an attempt to clone a *Rana pipiens* frog
- 1996 – the first mammal cloned from a cell taken from an adult animal – Dolly the sheep
- 1998 – the first cloned mouse (it was called Cumulina)
- March–April 2003 – a rabbit is cloned in France and Southern Korea
- 2009 – the first animal from an extinct species cloned: Pyrenean ibex. The animal lived seven minutes. It died of lung malformations

Types of Cloning:

1. Recombinant cloning
2. Therapeutics cloning
3. Reproductive cloning

Recombinant Cloning: Recombinant DNA (rDNA) molecules are DNA molecules formed by laboratory methods of genetic recombination to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in the genome.

Cloning Strategies:

The cloning strategy is divided into four stages :

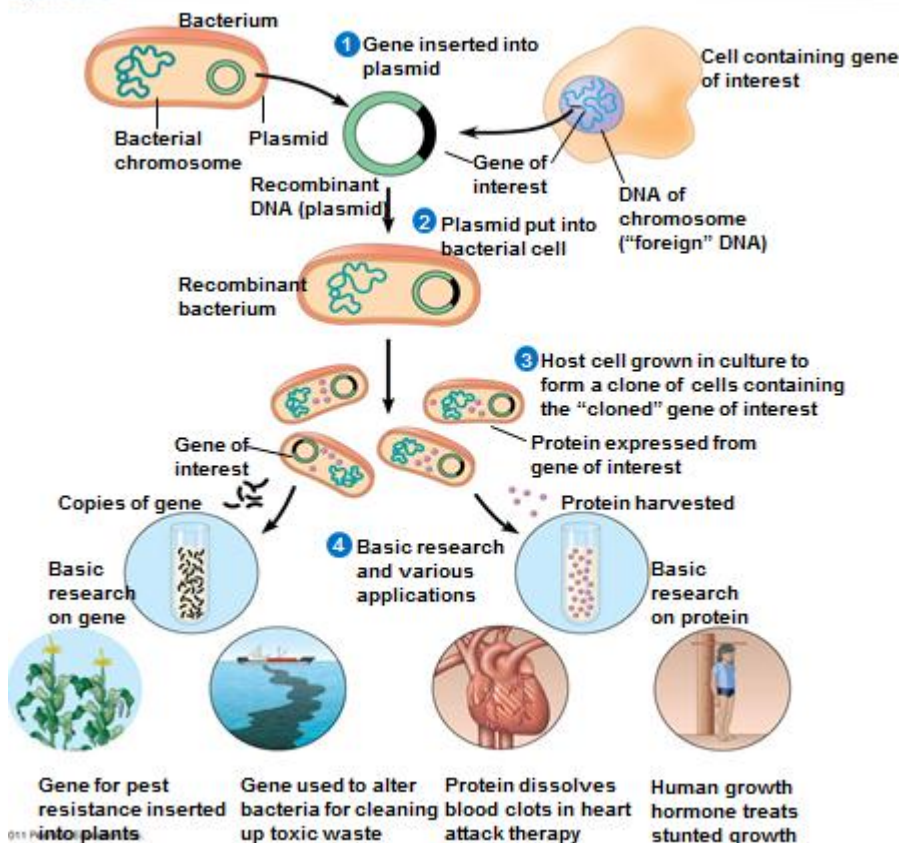
- Obtaining DNA fragments
- Joining to vectors
- Introduction into host cell
- Screening or Selection

Cloning Tools

- Endonucleases
- Ligases
- Vector
- Host

- Transformation

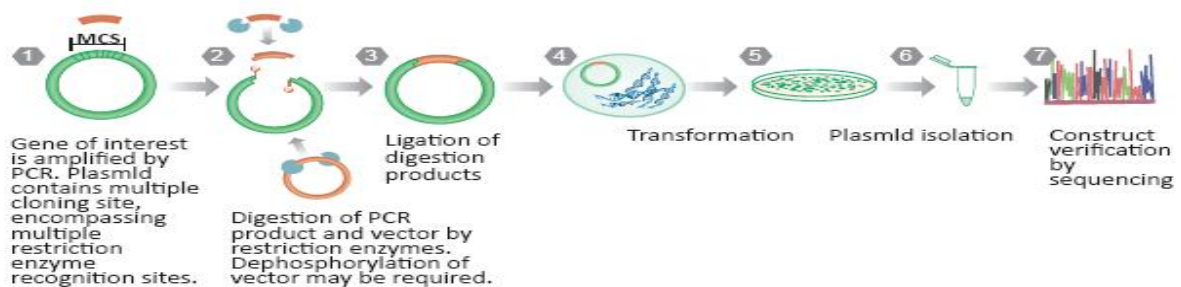
Figure 20.2



Types of Recombinant Cloning

- Traditional cloning
- TA cloning
- Seamless cloning

Traditional cloning, requires the use of the polymerase chain reaction (PCR) to amplify the template sequence of interest and add restriction sites to the ends of the sequence.



TA cloning is one of the simplest forms of cloning. In this method, vectors containing 5' thymine overhangs are used to accept PCR products in which additional 3' adenosine overhangs have been added on by the nature of TAQ polymerase amplification.

Seamless cloning technologies eliminate the requirement for restriction enzymes. This can be advantageous when an insert contains a number of restriction sites within its sequence, making it difficult to identify restriction enzymes that will not cut the gene of interest during the cloning procedure.

Benefits:

- Many copies of genetic material available in the laboratory for the purpose of experimentation
- Production of pharmaceutical drugs, insulin, clotting factors, human growth hormone, and industrial enzymes
- Important for human research
- For production of GMOs

Reproductive Cloning:

- The production of a genetic duplicate of an existing organism
- Somatic cell nuclear transfer

Therapeutic Cloning: Cloning designed as therapy for a disease. In therapeutic cloning, the nucleus of a cell, typically a skin cell, is inserted into a fertilized egg whose nucleus has been removed. The nucleated egg begins to divide repeatedly to form a blastocyst.

113. GRAFTING TECHNIQUES

Grafting: Grafting refers to a surgical procedure to move tissue from one site to another on the body, or from another person, without bringing its own blood supply with it. Instead, a new blood supply grows in after it is placed. In some instances a graft can be an artificially manufactured device. Examples of this are a tube to carry blood flow across a defect or from an artery to a vein for use in hemodialysis.

Classification

AUTOGRAFT: graft taken from one part of the body of an individual and transplanted onto another site in the same individual, e.g., skin graft.

ISOGRAFT: graft taken from one individual and placed on another individual of the same genetic constitution, e.g., grafts between identical twins.

ALLOGRAFT: graft taken from one individual placed on genetically non-identical member of the same species.

XENOGRAFT: graft taken from one individual placed on an individual belonging to another species, e.g., animal to man.

Types of Grafting:

The term grafting is most commonly applied to skin grafting. Specific types include:

- Skin grafting
- Bone grafting
- Tendon grafting
- Cartilage grafting

1. Skin Grafting: A skin graft is a procedure performed where healthy skin is removed from one area of the body, the donor site, and transplanted to another, the recipient site. The areas of the body that are most commonly used as donor sites for skin graft are the legs, inner thigh, upper arm and fore arm.

Types of Skin Grafting

1. Split thickness graft

2. Full thickness graft

3. Composite skin graft

- **Split or Partial Thickness Graft :** This is the most common type of graft. The epidermis and part of dermis are removed from donor site and transplanted on the damaged area.
- **Full Thickness Graft:** The entire epidermis and dermis are transplanted to the recipient site. The donor site needs to be either closed with stitches or have a partial thickness graft transplanted.
- **Composite Skin Graft:** A small graft containing skin and underlying cartilage or other tissue. Usually skin and fat or skin cartilage often taken from ear margin and useful for rebuilding missing elements of nose, eyelids and fingertips.

2. Bone Grafting: Used for the replacement or augmentation of the bone . Used in dental implants, as well as other instances. The bone may be autologous, typically harvested from the iliac crest of the pelvis, or banked bone.

Types of Bone Grafting

1. Bone blend
2. Bone swagging

- **Bone Blend:** Uses an autoclave capsule and pestle. Bones removed from predetermined site, triturated in capsule in a workable plastic like mass and packed in a bony defects.
- **Bone Swagging:** Techniques required existence of an edentulous area adjacent to the defect from which bone is pushed in contact with root surface without fracturing the bone at its base
- 3. **Tendon Graft:** Contraindicated if there is stiff joints, adherent extensor tendons, and inadequate skin cover. Only autograft. Unacceptable amount of host reaction and adhesion after allografts and xenografts

4. Cartilage Graft: Cartilage has no intrinsic blood supply. The use of cartilage autografts is widespread and includes nasal, auricular, craniofacial skeleton, and joint reconstruction. Both chondrocytes and matrix are subject to xenogeneic mechanisms of rejection with a generally poorer outcome in comparison. There is only small number of usage.

114. GENOME WIDE ASSOCIATION STUDY (GWAS)

A genome-wide association study (**GWAS**) is an approach used in genetics research to associate specific genetic variations with particular diseases. The method involves scanning the genomes from many different people and looking for genetic markers that can be used to predict the presence of a disease.

Central Goal of Human Genetics

- To identify genetic risk factors for common, complex diseases
- Goal of GWAS to use genetic risk factors to predict who is at risk
- Identify the biological underpinnings of disease susceptibility for developing new prevention and treatment strategies

Concepts underlying the study design

- SNP---single nucleotide polymorphism
- Single base pair changes in the DNA sequence that occur with high frequency in the human genome
- SNP (common) vs. Mutation (rare)
- Cystic fibrosis---mutations in the CFTR gene
- Linkage analysis---genotyping families affected by cystic fibrosis using a collection of genetic markers across the genome and examining how these genetic markers segregate with the disease across multiple families

Common Disease Common Variant Hypothesis: Common disorders are likely influenced by genetic variation that is also common in the population

- I. If common genetic variants influence disease, the effect size (or penetrance) for any one variant must be small relative to that found for rare disorders.
- II. If common alleles have small genetic effects (low penetrance), but common disorders show heritability (inheritance in families), then multiple common alleles must influence disease susceptibility.

Capturing Common Variation: Location and density of commonly occurring SNPs is needed to identify the genomic regions and individual sites that must be examined by genetic studies. Population-specific differences in genetic variation must be cataloged so that studies of phenotypes in different populations can be conducted with the proper design. Correlations among common genetic variants must be determined so that genetic studies do not collect redundant information

International Hap Map Project: Used a variety of sequencing techniques to discover and catalog SNPs in European descent populations, the Yoruba populations of African origin, Han Chinese individuals from Beijing, and Japanese individuals from Tokyo. Has since been expanded to include 11 human populations

Linkage Disequilibrium: A property of SNPs on a contiguous stretch of genomic sequence that describes the degree to which an allele of a SNP is inherited or correlated with an allele of another SNP within a population. Linkage between markers on a population scale

Direct vs. Indirect Association: LD creates two possible positive outcomes from a genetic association study

- Direct association----the SNP influencing a biological system that leads to the phenotype is directly genotyped in the study
- Indirect association----the influential SNP is not directly typed, but instead a tag SNP in high LD with the influential SNP is typed

Therefore, a significant SNP association from a GWAS should not be assumed as the causal variant

Application in Pharmacology:

- Identifying DNA sequence variations associated with drug metabolism and efficacy as well as adverse effects. Example, warfarin---determining the appropriate dose
- Personalized medicine

115. NUCLEIC ACID AND CELL BASED THERAPIES

“Therapeutic techniques and methods that have a curative approach while using nucleic acids(DNA/RNA) and certain cells of the body.”

Nucleic acid based:

Cell based therapies: Stem cell

- Gene therapy
- Antisense technology
- Aptamers
- RNAi

Gene Therapy: The stable introduction of a gene into the genetic complement of a cell, such that subsequent expression of the gene achieves a therapeutic goal. The potential of gene therapy as a curative approach for inborn errors of metabolism and other conditions induced by the presence of a defective copy of a specific gene (or genes) is obvious.

- Cancer, AIDS, cystic fibrosis, SCID, familial hypercholesterolemia etc. are under clinical trials.
- Requirements for the introduction of gene of interest
- Vector (viral or non-viral)
- Cell line (opted according to the need)

Antisense Technology: Antisense therapy is a form of treatment for genetic disorders or infections. When the genetic sequence of a particular gene is known to be causative of a particular disease, it is possible to synthesize a strand of nucleic acid (DNA, RNA or a chemical analogue) that will bind to the messenger RNA (mRNA) produced by that gene and inactivate it, effectively turning that gene "off".

Aptamers: Aptamers are single-stranded DNA or RNA-based sequences that fold up to adopt a unique three dimensional structure, allowing them to bind a specific target molecule.

- Highly specific , high binding affinity
- DNA and RNA based aptamers libraries synthesized by chemical methods.
- Only one product available in market by the name of “Macugen”.

RNA Interference (RNAi): Biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules via RISC(RNA Induced splicing complex).

- A method of gene silencing
- Viral infections
- Cancer therapy

Cell Based Therapies (Stem Cells)

On the basis of source:

1. Adult (adipose tissue, blood, bone marrow, umbilical cord)

2. Embryonic (pre implant stage embryos)

This technique is used in:

- Transplants
- Metabolic diseases (diabetes)
- Cancer therapy
- Induced pluripotent cells

Advantages

- Less chances of adverse effects
- Low mortality rate
- Cheaper and reliable
- AIDS and various cancer treatment trials are in process.

Disadvantages

- Sometimes immunosuppression is required
- Tumor formation is common in transplantation
- In case of gene therapies, multigene disorders can't be addressed through this technique.

Current Status: Only three nucleic acid based medicines in market. one antisense-based product (trade name Vitravene), one aptamer (trade name Macugen) and one gene therapy product (trade name Gendicine, approved only in China).

- Till now, 7 deaths are reported during the clinical trials.
- 1200 clinical trials of gene therapy carried out worldwide.

116. RECOMBINANT HORMONES

Hormone: Hormones are type of growth factors, they act as a messenger between cells and are vital for the function of multi-cellular organisms 

Recombinant Hormones: Synthetic hormones which are being produced by genetic engineering rather than extracting them from animals and humans. e.g. HGH, Insulin, FSH

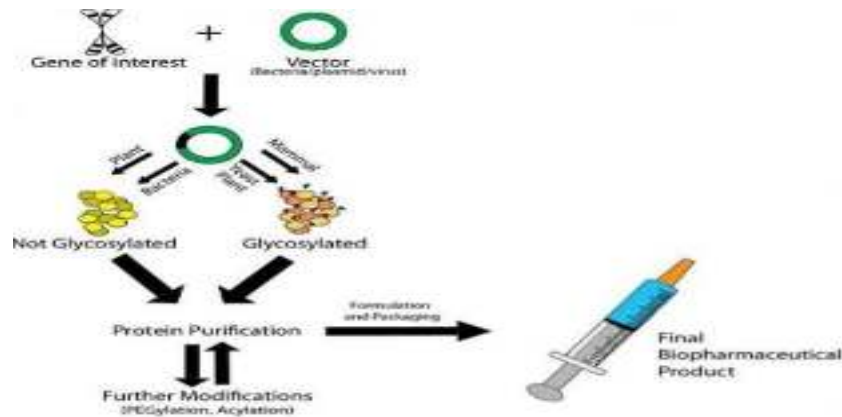
Endocrine System: The endocrine system is made up of glands that produce and secrete hormones.

Hormones regulate:

- The body's growth

- Metabolism
- Sexual development and function

Recombinant Hormone Production



List of Recombinant Hormones

- Human growth hormone (rHGH)
- Biosynthetic human insulin (BHI)
- Factor VIII
- Erythropoietin (EPO)
- β -ENDORPHIN
- Follicle-stimulating hormone (FSH)
- Bovine somatotropin (bST)
- Interferon (IF)
- Somatostatin

Recombinant Hormones	Strain / Cell line used	Function / Role
Insulin	E. coli	Hormone which lowers blood sugar used by diabetics
Human Growth hormone	E. Coli CHO cells	For normal body growth & development
Erythropoietin	CHO cells	production of red and white blood cells
Factor VIII	CHO or BHK cells	Blood protein necessary for clotting
β -ENDORPHIN	Mouse pituitary cell line	Pain suppressors
Bovine somatotropin (bST)	E. coli	Regulate metabolic processes. Enhance milk production
Somatostatin	CHO	Regulate endocrine system

Structure of Insulin

- Chemically, insulin is a small, simple protein
- Preproinsulin=108 amino acid
- 23 a.a signal peptide.
- 34 a.a long pro-part of the insulin is removed in secretory granules.
- Mature insulin consists of 51 amino acid, 30 of which constitute B-chain
- 21 of which comprise A-chain.
- The two chains are linked by a disulfide bond

Protein Extraction and Purification

- Cell disruption
- Centrifugation
- Ultra filtration
- Diafiltration
- Chromatography
- Addition of excipients
- Sterile filtration
- Powder or liquid preparation

- Sealing, labelling and packing of final product

Human Growth Hormone/ Somatotropin

- Secreted by the pituitary gland
- Responsible for normal body growth and development
- Stimulate protein production in muscle cells
- Energy release from the breakdown of fats
- Stimulates the development of bones
- **Growth Hormone:** 191 amino acids, single chain

Erythropoietin: A glycoprotein, acts on the bone marrow to increase the production of red and white blood cells. Stimuli such as bleeding or moving to high altitudes (where oxygen is scarce) trigger the release of erythropoietin. Kidney is the principal production site of Erythropoietin. Kidney cells specifically sense the oxygen deficit in the blood and start producing Erythropoietin

Recombinant hormones now approved

- Glucagon
- Thyroid stimulating hormone
- Parathyroid hormone
- calcitonin

117. REPRODUCTIVE TECHNOLOGIES

Infertility: Inability to conceive after 1 year of properly timed unprotected intercourse. It is world wide problem effecting 13-14% of reproductive –aged couples.

Infertility may be

- Primary, where no history of pregnancy ever having occurred
- Secondary, inability to conceive after one successful pregnancy or after an abortion

Causes of Infertility

Female

- Dietary disturbances, anxiety, fear etc.
- Uterine absence, hypoplasia etc.
- Pituitary failure, thyroid disturbances, polycystic disease.

- Pelvic inflammation, T.B. etc.

Male

- Fatigue, excess smoking, alcohol etc.
- Undescended testis, other abnormalities
- Pituitary failure, thyroid deficiency etc.
- Mumps, arthritis, venereal disease etc.

Assisted Reproductive Technologies (ART): ART include those infertility treatments in which both eggs and sperms are handled in the lab for the purpose of establishing a pregnancy. Birth of Louise Brown in 1978, world's first test tube baby.

Various Assisted Reproductive Techniques

- Intra Uterine Insemination (IUI)
- Intracytoplasmic sperm injection (ICSI)
- In Vitro Fertilization (IVF)
- Gamete Intra Fallopian Transfer (GIFT)

Intra Uterine Insemination (IUI):

Suggested for subtle male factor infertility and an abnormal sperm mucus interaction

Procedure

- One million motile sperms are usually placed at the top of uterine cavity near the opening of the fallopian tubes.
- The whole procedure takes place during the fertile days i.e.13-15 of her menstrual cycle.

Complications

- Uterine perforation vary rare.
- Infection during insemination.
- Localized trauma of the endometrium can cause bleeding but has no risk
- Discomfort can cause uterine cramp.

Intracytoplasmic sperm injection (ICSI)

Suggested for men incapable of reproduction

Procedure

- The ovum is injected with single sperm in vitro with the help of micromanipulation devices.
- After the procedure the oocyte is placed into cell culture and checked for signs of fertilization.
- Once fertilization is accomplished, cell division occur and embryo is transferred into women's uterus.

Complications

- Naturally competent cells initiate fertilization which does not occur in in-vitro.
- Hyaluronan is used as a drop in the plate and sperms compete for it. Those sperms are selected and used for fertilization

In Vitro Fertilization (IVF)

This technology is developed to overcome the infertility due to the problems of:

- Couples with endometriosis, drug resistant polycystic ovarian syndrome etc.
- Age related ovarian dysfunction, ovarian failure or surgically removed ovaries
- Blocked fallopian tubules, ovulation problem
- Seminal antibodies in women
- Combine ICSI and IVF can be used to treat sperm disorders.

Four stage procedure

Stage 1- Ovarian stimulation and monitoring

Stage 2- Egg retrieval

Stage 3- Fertilization

Stage 4- Embryo transfer

Procedure

1. Women is given drugs to produce super-ovulation i.e. produces many eggs
2. Fluid collected from fallopian tubes and eggs separated
3. Semen sample taken at the same time.
4. Eggs and semen are placed in a culture dish to fertilize under special conditions.

5. Fertilization observed in the laboratory.
6. After three days of fertilization each successful embryo has divided to 8 cell level.
7. At this time three healthy embryos are transferred to the uterus.
8. Patient is given drugs to help support implantation
9. Generally after 12 to 14 days after embryo transplantation blood test is done to confirm pregnancy.

Complications

- Hyperstimulation syndrome
- Estrogen and kallikrein-kinin and vascular endothelial growth factor (VEGF) is poured from ovaries into abdominal cavity.
- These coat the lining of abdominal cavity and make it permeable i.e. leaky.
- Serum from blood pours into the cavity resulting balloon ovaries, abdomen swells, low blood pressure and may get dizzy due to it.

Cure

- Drainage of extra fluid
- Intravenous fluid intake

Gamete Intra Fallopian Transfer (GIFT): Developed in 1983, by Dr. Ricardo Asch. Suggested for woman with unexplained infertility or infertility due to cervical or immunological factors, mild endometriosis. In GIFT mixture of sperm and egg is injected into mother's fallopian tube where fertilization occurs. Rate of pregnancy is higher though as compared to other methods i.e. IVF and ICSI

Step 1. Over stimulation and monitoring

Step 2. Egg retrieval

Oocyte is aspirated either by transvaginal with ultra sound guidance or transabdominally by laparoscopic guidance.

Step 3. Gamete transfer

Sperm and egg are combined and incubated for 10 min. to allow sperm binding. 25,000 sperms and 2 oocyte are placed in fallopian tube with exogenous progesterone.

Limitations:

- Abnormal fallopian tubes not eligible.
- GIFT requires surgery for gamete transfer.

118. REPRODUCTIVE TECHNOLOGIES

Zygote Intrafallopian Transfer (ZIFT): Oocyte is retrieved from the ovary as in GIFT. Fertilization occurs in laboratory. After 24 hrs. 2-3 embryos are transferred via laparoscopy into one of the fallopian tubes. If embryos are allowed to grow greater than 2 cell stage then its called tubal embryo transfer (TET). Only benefit ZIFT over IVF is for women who are thought to have compromised embryo quality due to embryo in vitro culture. Placing zygotes back into their own natural incubators is thought to enhance development and improve pregnancy.

Blastocyst culture and Transfer:

For this

1. Have 3 or more IVF cycles and no pregnancy
2. Over 40 years of age
3. Want single pregnancy
4. Undergoing preimplantation genetic disorders.

Procedure

- Embryos are grown for 5 days until blastocyst stage and then transplanted. This ensures
- Max. pregnancy rates
- Minimizing the risk of multiple pregnancies.
- Good healthy blastocyst are transplanted via catheter. Remaining are frozen for further use.
- Occasionally cover of blastocyst is dissolved by an enzyme this increases the chance of implantation.

Preimplantation Genetic Diagnosis PGD: PGD improves the likelihood of successful pregnancy and is requested by prospective parents who are concerned about passing an incurable genetically based disease or disorder to their child. First PGD baby was born in 1989 and by 1997 over 30 babies were born worldwide. PGD can monitor 2 dozen disorders.

In Vitro Maturation (IVM): In vitro maturation (IVM) is the technique of letting the contents of ovarian follicles and the oocytes inside mature in vitro. It can be offered to women with infertility problems, combined with IVF, offering women pregnancy without ovarian stimulation. In vitro maturation of ova and less risk of over stimulation of mother.

- Gn-Rha (prevent ovulation till maturation)
- FSH (stimulate multiple follicle production)

- HCG (final maturation of eggs)

119. TYPES OF GENE THERAPY

Somatic gene therapy: Transfer of a section of DNA to any cell of the body that doesn't produce sperm or eggs. Effects of gene therapy will not be passed onto the patient's children.

Germ line gene therapy: Transfer of a section of DNA to cells that produce eggs or sperm. Effects of gene therapy will be passed onto the patient's children and subsequent generations

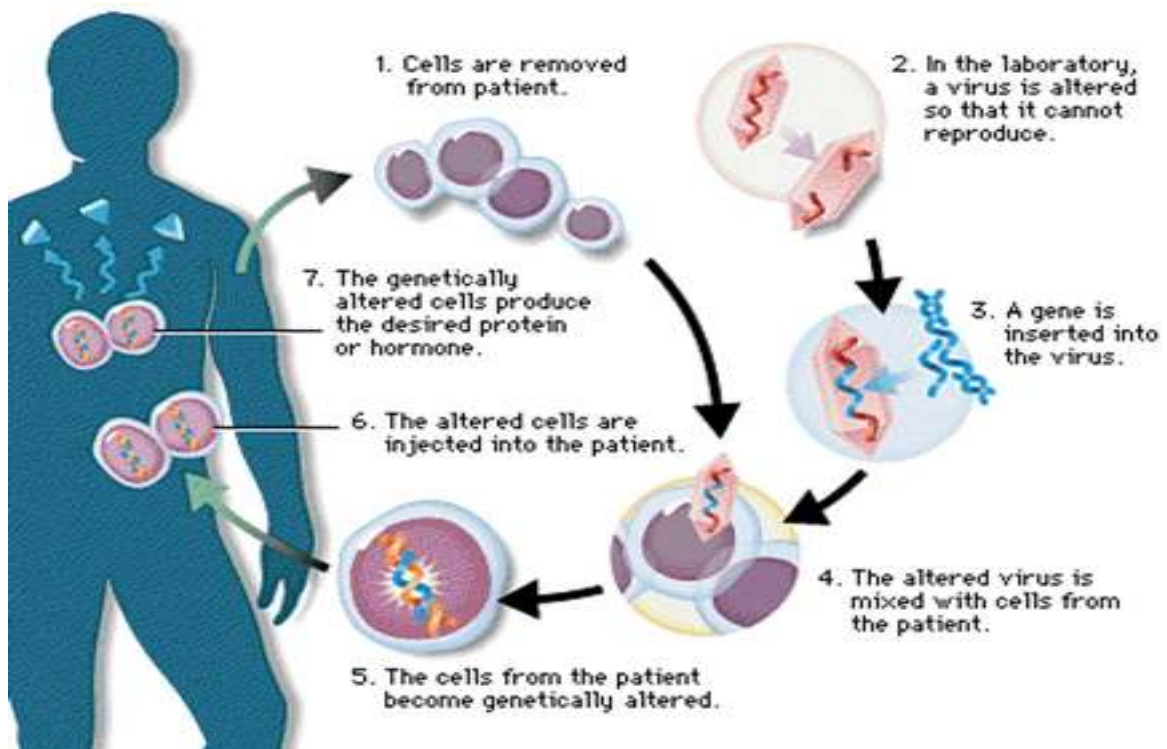
Somatic Gene Therapy Types

1. In vivo Gene Therapy

- Delivery of new Genetic material directly to target cells in the body
- Transient expression
- Treatment of cystic fibrosis
- Lungs ,Brain

2. Ex vivo Gene Therapy

- Cells are modified outside the body and then transplanted back it again
- Long-term expression
- Fibroblast cells, Hematopoietic cells



GENE AUGMENTATION THERAPY

This is used to treat diseases caused by a mutation that stops a gene from producing a functioning product, such as a protein. This therapy adds DNA containing a functional version of the lost gene back into the cell. The new gene produces a functioning product at sufficient levels to replace the protein that was originally missing. This is only successful if the effects of the disease are reversible or have not resulted in lasting damage to the body.

For example, this can be used to treat loss of function disorders such as cystic fibrosis by introducing a functional copy of the gene to correct the disease

GENE INHIBITION THERAPY

Suitable for the treatment of infectious diseases, cancer and inherited disease caused by inappropriate gene activity. The aim is to introduce a gene whose product either:

- inhibits the expression of another gene
- interferes with the activity of the product of another gene.

The basis of this therapy is to eliminate the activity of a gene that encourages the growth of disease-related cells. For example, cancer is sometimes the result of the over-activation of an oncogene (gene which stimulates cell growth). So, by eliminating the activity of that oncogene through gene inhibition therapy, it is possible to prevent further cell growth and stop the cancer in its tracks.

Risk of Gene Therapy

This technique presents the following risks:

- 1. Unwanted immune system reaction.** Your body's immune system may see the newly introduced viruses as intruders and attack them. This may cause inflammation and, in severe cases, organ failure.
- 2. Targeting the wrong cells.** Because viruses can affect more than one type of cells, it's possible that the altered viruses may infect additional cells — not just the targeted cells containing mutated genes. If this happens, healthy cells may be damaged, causing other illness or diseases, such as cancer.
- 3. Infection caused by the virus.** It's possible that once introduced into the body, the viruses may recover their original ability to cause disease.
- 4. Possibility of causing a tumor.** If the new genes get inserted in the wrong spot in your DNA, there is a chance that the insertion might lead to tumor formation.

120. DNA FINGERPRINTING

DNA Fingerprinting is a way to identify a certain individual, rather than simply identifying a species or a particular trait. A technique used by scientists to distinguish between individuals of the same species using only samples of their DNA. The process of DNA fingerprinting was invented by Alex Jeffreys in 1985.

Biological Sample used for DNA Fingerprinting

- Blood
- Hair
- Saliva
- Semen
- Body tissue cells

Steps to DNA Fingerprinting

There are 8 steps for DNA Fingerprinting

Step 1: Isolation of DNA

- DNA must be recovered from cells or tissue.
- Only a small amount of blood, hair, or skin is needed to isolate DNA

Step 2: Digestion of DNA by restriction endonucleases

- The DNA is cut into fragments using restriction enzymes
- Each restriction enzyme cuts DNA at a specific base sequence
- The sections of DNA that are cut out are called restriction fragments
- This yields thousands of restriction fragments of all different sizes because the base sequences being cut may be far apart (long fragment) or close together (short fragment)
- Commonly used RE are (hae III, Hinf I, Alu I etc.)
- Reaction mixture is incubated overnight at 37C

Step 3: Electrophoretic separation of different fragments

- Fragments are separated on the basis of size using a process called gel electrophoresis
- DNA fragments are injected into wells and an electric current is applied along the gel.
- DNA is negatively charged so it is attracted to the positive end of the gel.
- The shorter DNA fragments move faster than the longer fragments

- DNA is separated on basis of size.

Step 4: Transfer DNA on Nylon/Nitrocellulose membrane

- The DNA fragments are transferred to a nylon sheet by placing the sheet on the gel and soaking them overnight by the process southern blot.

Step 5: Probing/probe labeling

- Adding radioactive or colored probes to the nylon sheet which is complementary to target sequences
- Each probe only sticks to one or two specific places on the sheet

Step 6: Hybridization

- Labeled probe DNA should be hybridized with the complementary sequences located on nylon membrane for the detection of position of later
- Membrane is washed to remove non specific binding and clearing of the background

Step 7: Autoradiography

- To detect the sequences in genome bound with the hybridized radioactive probe on membrane
- Technique involves alignment of hybridized membrane with X-ray film
- The X-ray film after its development shows multiple no. of bands that look like bar codes and known as DNA fingerprints.

Step 8: Interpretation of band patterns

- Analysis of band patterns of different individuals
- Comparison of position of bands
- Computer software is also available for the analysis of DNA fingerprints.

USES

Diagnosis of Inherited Disorders

- Helps diagnose disorders in both prenatal and newborn babies
- Disorders may include cystic fibrosis, hemophilia, Huntington's disease, familial Alzheimer's, sickle cell anemia, thalassemia, and much more.

Crime

- Forensic science is the use of scientific knowledge in legal situations.

- The DNA profile of each individual is highly specific.
- The chances of two people having exactly the same DNA profile is 30,000 million to 1 (except for identical twins).

Personal Identification

- The U.S. armed services are just beginning a program where they collect DNA fingerprints from all personnel for later use, incase they need to identify casualties or missing people

Identification of carcass of tissues

Detection of somatic mutations or cancer

Pathogen identification

Detection of loci controlling quantitative traits or disease resistance

Sex determination

Individual identification