1. BIOINFORMATICS

Bioinformatics ((derived from bio-biology, informatique-French for data processing) is one of the fastest growing field of study that has diverse fields merged into a single discipline. The diverse fields include biology, computer science, information technology, molecular biology, medicine, biotechnology, pharmacology, agriculture and related bioscience done by teams of biologists, chemists, computer scientists, information technologists, mathematicians, physicist as well as statisticians. The synonyms of bioinformatics are computational biology, computational molecular biology and biocomputing.

Bio informatics is considered to be an epitome of modern scientific research. It involves the use of computers to search for, explore and use information about genes, nucleic acids and proteins.

Purpose:

Before the evolution of computers, biologists would perform an experiment in the laboratory under controlled conditions, which is known as *in vitro* or in glass. Another way to study the problem was to perform experiment in a living being – for instance in a guinea pig, which is known as *in vivo* or in life. And yet, another way to perform experiment is *in silico* or *in silicon*, wherein simulated experiments are conducted with a computer. This makes the experimenting faster, easier and less expensive. This way, bioinformatics has evolved to compensate for the lack of better ways store and analyze important data. The results of the experiment can then be tested *in vitro* or *in vivo*.

The development of DNA sequencing technology has enabled the sequencing of a number of small genomes. The DNA sequence of a human bacterial pathogen, *Haemophilus inflenzae* was published in 1995. The other organisms whose genomes have been fully sequenced and published are *Mycoplasma genitalium, methanococcus jannaschii, Mycoplasma penumoniae, Sacharomyces cerevisiae, Escherichia coli* K-12, *Helicobacter pylori* and many more.

A human cell has 3×10^9 base pairs (3 billion) in two DNA strands that form one double helix like a twisted ladder. Every base pair forms a step in the twisted ladder. The distance between the two strands is 0.34 nm. It is coated with proteins which untwist the helix to allow gene expression and wind it up into tightly packed supercoils.

The information about our DNA, proteins and their functions are stored in an intelligent fashion and for that we have computers for storing large amount of data in digital format. The Human Genome Project (HGP) could not have been possible without the help of computers. All the human genes have been discovered and made accessible for further biological study.

Biological Database/Molecular Biology Database

Data, in bioinformatics, is the information about genes and proteins as well as their sequences. A biological database houses and securely stores huge, organized data associated with computer software which can be updated, and queried within the system. The data stored in a database should be easily accessible as well as provide information regarding a specific biological question.

Databanks store information about genes and proteins, some of which are accessible to us on the internet. This has enabled researchers to carry out a multitude of research and clinical applications.

A few examples of databases are given below:

1. **GenBank (Genetic Sequence Databank):** GenBank files contain information about the sequences of DNA and their gene products. It is linked to other databases fo information about genes. The database has ASCII text file which can be read by both humans and computers. GenBank is operated by NCBI (National Center for Biotechnology Information).
2. **EMBL (European Molecular Biology Laboratory):** This databank has nucleotide sequences of all genes sequenced throughout. EMBL is the European version, while GenBank is the American version. Both these databanks synchronise their databases and contain the same information.
3. **DDBJ (DNA Databank of Japan):** Both EMBL and DDBJ contain the same DNA sequence content as NCBI.
4. **OMIM (Online Mendelian Inheritance in Man):** OMIM contain information on human genes and genetic disorders. This databank is linked to GenBank and PubMed for updated information on scientific literature.
5. **PDB (Protein Data Bank):** It has information on the structure of proteins and nucleic acids that have been experimentally determined by X-ray crystallography andNMR.
6. **PubMed:** PubMed is the search engine for scientific literature on biomedical research.
7. **Uniprot Knowledge base (Swiss-Prot and TrEMBL):** This database has information on sequences of proteins. It is operated by SIB (Swiss Institute of Bioinformatics) and EBI (European Bioinformatics Institute). Swiss prot is a databank containing protein sequences and their function.

Tools of Bioinformatics:

The different types of databanks mentioned above can perform all kinds of comparisons and search queries, only with the help of tools. Tools are software programs that are meant for extracting useful and meaningful information.

1. **BLAST (Basic Local Alignment Search Tool):** This is used to search database to find genes or proteins with similar sequences as that of a particular subject, say X.
   It has different programs:
   a. **Blast p:** This programme is used to compare an amino acid query sequence against a protein sequence database.
   b. **Bast n:** This programme is used to make a comparison between nucleotide query sequence and nucleotide sequence database.
   c. **Balst x:** This process is used for comparison between nucleotide query sequence and a protein sequence database.
2. **Clustal W:** It is an automated sequence alignment programme used for DNA and protein sequences.
3. **Deep View (Swiss Pdb Viewer):** This tool is used for viewing models of macromolecules in there dimensions.

4. **ExPASy (Expert Protein Analysis System):** This tool is used for analysis of proteins.
5. **NCBI Map Viewer:** This tool is used for finding genes and gene products (i.e. RNAs and proteins).
6. **PubMed:** The tool is used for searching scientific literature on life sciences.
7. **Tcoffee (Tree based Consistency Objective Function for Alignment Evaluation):** This tool is used for sequence comparisons. It is a multiple sequence alignment package, can align proteins DNA and RNA sequences.
8. **EMBOSS (European Molecular Biology Open Software Suite)**
9. **COPIA (Consensus Pattern Identification and Analysis):** It is a protein structure analysis tool for studying the conserved regions in a family of protein sequences.

   Thus, it can be said that bioinformatics is a significant area of life sciences and will continue to develop and support our research and understanding of DNA and the biology of organisms.

Applications of Bioinformatics: The world bioinformatics has opened its doors for finding new applications at all times. Some of the many applications of bioinformatics are discussed below.

1. **Molecular Medicine:**

   The sequencing of human genome has enabled us to understand the genes that are directly associated with different diseaces and analyze the molecular basis of disease.

   a. **New Drug Targets for Specific Drugs:** The pharmaceutical industry ca manufacture drugs that can target a specific metabolic pathways involved in the disease. It is hoped that this new generation of drugs will work better and cause fewer side effects.
   b. **Personalized Treatment:** The medical treatment is likely to become more personalized with the advent and development of the field of pharmacogenomics, a study involved in the methodologies to target drugs specifically to these patients with genetic profile such that they will have close to 100% response with no side effects.
   c. **Preventive Medicine to Different Diseases:** Based on the information contained in the genome, clinicians can develop personalized strategies for detecting, treating and preventing diseases. For instance, if the genetic profile shows that there is an increased risk for colon cancer, the healthcare providers would advice such patients to undergo frequent colonoscopy screening and reduce the intake of meat and diet. Similarly, if the genome shows that there is a risk of heat disease, then the patient would be advised to exercise more and take drugs that lower the cholesterol content in the blood.

      According to the latest reports, specialized sophisticated gene tests are conducted to screen genetic flaws among embryos used in *in vitro* fertilization. This is called preimplantation genetic diagnosis (PGD). Such a test enables the selection of only mutant-free embryos for implantation into the mother's uterus.
   d. **Gene Therapy:** Gene therapy involves inserting normal gene into the genome to replace the abnormal disease causing gene with the help of vector. Some of the vectors used in gene therapy are retroviruses, adenoviruses,adeno-associated viruses, herpes simplex virus. Other non-viral options for therapeutic gene delivery are direct introduction of

therapeutic DNA into target cells, and by using liposomes (artificial lipid sphere with an aqueous core).

2. **Applications in Environment, Health, Energy and Industry:**

Microbes are present everywhere. They can thrive in an amazing diversity of habitats with extremes of temperatures, radiation, pressure, salinity, acidity as well as in darkness.

Microbial Genome Project (MGP) undertaken by the US Department of Energy is involved in the study of genetic material of microorganisms and their sequences that are useful in energy production, waste cleanup in the environment, and toxic waste reduction.

Microbial Genome Project (MGP) scientists are working on DNA sequence of the genome of *Deinococcus radiourans* and *C. crescentus.* The former is useful in cleaning up waste sites that contain radiation and toxic chemicals while the latter is used in bioremediation of contaminated environments. They can destroy contaminants of petroleum in the polluted groundwater by oxidizing the contaminants to carbon dioxide, *Geobacter species* can also be used in generation of electricity from waste organic matter.

The genome of the microbe *Chlorbium tepidum* is being studied for its ability to generate energy from light. *Archaeoglobus fulgidus* and *Thermotoga maritina* produces heat-stable enzymes useful in various industrial processes. The genome of *Corynebacterium glutamicum* is of interest to the scientists for the industrial production of essential amino acid lysine.

Researchers are interested in the physiology and genetic makeup of *Xanthomonas campestris* and *Lactococcus lactis,* the latter is used in dairy industry (manufacture of buttermilk, yogurt, cheese) and also to prepare pickles, beer, wine, breads, sausages and other fermented foods.

The DOE Carbon Management Program (USA) have sequenced the genome of *Rhodopesudomonas palustirs* for its ability in the management of environmental carbon dioxide related to climate change. It can convert carbon dioxide into cell material, nitrogen gas into ammonia, sunlight into cellular energy, produce hydrogen gas, degrade and recycle aromatic compounds containing lignin.

3. **Applications in Agriculture:** Bioinformatics is used in many aspects of agriculture for instance to improve nutrient quality of foods, developing crops that can grow in poor soil producing conditions as well as hard weather.

Two species of food plans for which the genomes has been completely mapped are *Arabidopus thaliana* (water cress) and *Oryza sativa* (rice). Water cress has a small genome size of 5 chromosomes containing 100 mbp DNA. It reproduces very quickly and makes new plants. The genomic sequence of his plant gives information about the plant proteins and its expression.

Plants can be made insect resistant by introducing desired genes. *Bacillus thuringiensis* is a bacterial species which increases the fertility of soil and protects the plant against harmful pests. Researchers have introduced the genes of *B. Thuringiensis* into plants (cotton, maize, potatoes) to make them insect resistant.

Scientists have introduced some genes into the rice genome to increase the levels of vitamin A, iron and other micronurtrients in the crop.

4. **Animal Husbandry:** Bioinformatics has a wide range of applications in animal husbandry. Genome sequencing of farm animals such as cows, pigs and sheep ar being carried out in research laboratories in the hope to widen the perspective of the biology of these organisms and improve their production and health.
5. **Bioinformatics for Comparative Studies:** various tools in bioinformatics can be used for comparative study of the numbers, locations and biochemical functions of genes in different organisms.

   The model organisms used for conducting research should have short life span, reproduce quickly and be inexpensive. The model organisms for humans in Mus musculus as studies have revealed that both have the same number of nucleotides in their genomes i.e. 3 billion base pairs. Alterations in the genes of the mouse genome followed by a comparative study between the two species provide wealth of information about the functions of human genes, evolutionary relationships and molecular mechanism of human diseases.

Other applications of bioinformatics include detections of disease causing organisms and monitoring of the safety of food and water supplies, use of genetically altered bacteria as living sensors to detect harmful chemicals in soil, air or water.

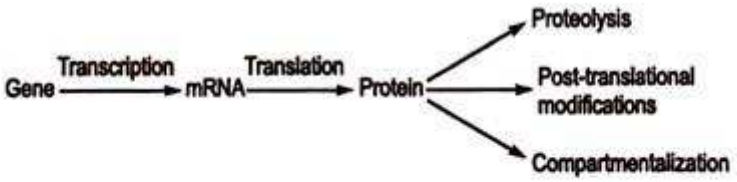
2. PROTEOMICS

Proteomics involve the study of all proteins and their interactions in a Cell or an organism.

The protein profile differs from one cell type to another and during different developmental stages. For instance the genetic profile of a Caterpillar and butterfly is the same but the protein profiles are different, thus giving them different morphology and function as if they are two different creatures.

The study of proteome is a challenging task especially when human genome is taken into consideration. Human genome containing about 35000 genes can code for huge number of proteins. Further the amino acids (the building blocks of proteins) are too small, each of them between 7 and 24 atoms. Hence this make researchers to determine the sequence of amino acids in a protein by breaking it into smaller pieces and then weigh each amino acid using a mass spectrophotometer.

I. Sequencing and identification of proteins:



Diagrammatic representation of a gene expression showing formation of many protein isoforms from a single gene. After transcription of the gene, mRNA is alternatively spliced or edited to form a mature mRNA that is translated to the protein. Proteins can be regulated by additional mechanism of proteolysis, compartmentalization and certain other modifications

The sequencing and identification of proteins is carried out in the following manner.

1. Millions of copies of a cell type are made followed by extraction of proteins. A single cell is grown in a medium which multiply to form many cells. Alternatively, a group of cells may be taken from biopsy or body fluids.
   The cells str scrapped off from the culture and transferred to a test tube containing buffer solution. A detergent solution is added to rupture the outer membrane of the cells. The test tube is spun in a centrifuge. The cellular membranes, cytoskeleton and other cell material pellet out at the bottom of the test tube. The supernatant contains the proteins.
2. **Separation of proteins using 2D-electrophoresis:** The proteins are placed in gel strip that has pH gradient from acidic to alkaline. When the voltage is applied, the proteins migrate on the strip according to their isoelectric point. The proteins are separated in a

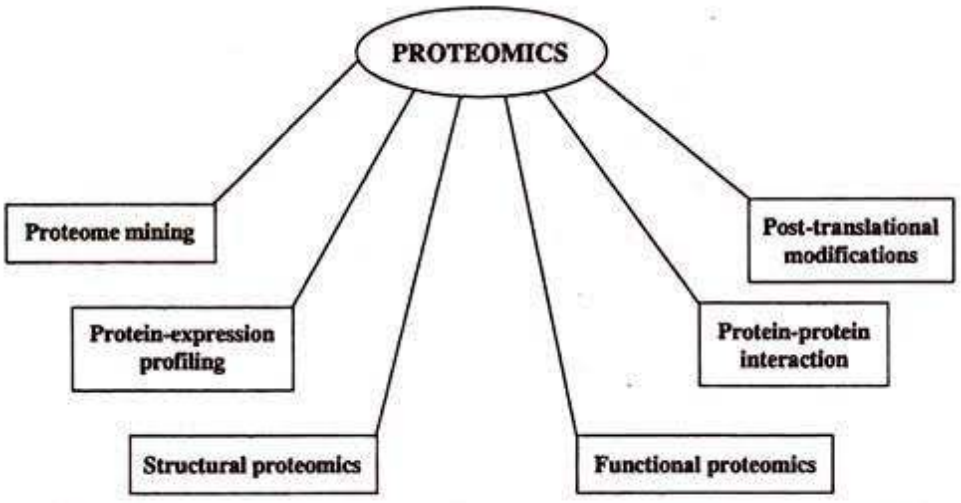
second dimension to sort them according to size. Small protein molecules migrate through the gel faster than large proteins.

3. **Transfer of proteins to a 96-well plate or a test tube:** Protease, an enzyme that cleaves proteins into peptides is added to the test tube. The mixture is allowed to dry. This is followed by addition of another solvent.
4. The solvent along with peptides is passed through liquid chromatography followed by mass spectrometer. The mass of individual peptides is determined in the mass spectrometer. The sequences of the fragmented peptides are compared with sequences stored in a database. This determines the identity of the protein.

II. Tools of Proteomics: The tools of proteomics include the following:

1. **Database:** Protein database has a complete catalog of proteins expressed in a particular organism. For instance, it is now known that 87 genes code for proteins with tyrosine kinase catalytic activity in *Drosophila.*
2. **Mass spectrometry:** This instrument provides accurate molecular mass measurements of intact proteins.
3. **Software:** The software packages related to proteomics can match mass spectrometry data with specific protein sequences in databases.
4. **Analytical protein separation techniques:** This technique enables separation of individual protein from a complex protein mixture. Other protein separation techniques include two dimensional dodecyl sulphate polyacrylamide gel electrophoresis (2D-SDS-PAGE), 1D-SDS-PAGE, High Performance liquid chromatography (HPLC), capillary electrophoresis (CE), isoelectric focusing (IEF) and affinity chromatography.

III. Applications of proteomics: the study of proteomics involves the identification of complete set of proteins synthesized by a cell in the body as well as determination of the role of identified proteins in transmitting diseace because all proteins are functional entities in a cell.



Understanding the applications of proteomics

The principle applications of proteomics are-

1. **Data mining:** Data mining is the process of searching protein databases, also known as **data ware houses** for efficient and accurate data analysis of biological specimens form healthy and diseased individuals.
2. **Protein-expression profiling:** It deals with identification of proteins in a specific sample as a funtionsof a particular state of the organism or cell or as a function of exposure to a drug or physical stimulus. Expression profiling is a form of high level mining. It commonly finds applications in differential analysis in which two states of a particular system are to be compared.
   For example, normal and diseased tissues/cells can express differently. This information is used to identify target sites in a protein where a drug can bind work.
3. **Protein Network Mapping:** It is the proteomics approach to differentiate how proteins interact with other proteins in living systems. These interactions determine the functions of protein functional networks.
   In vitro studies are expensiv, time consuming, as well as restricted to individual proteins like yeast cells. Proteomics is capable of studying and characterizing complex protein networks. Affinity capture techniques along with analytical proteomic methods are powerful tools to facilitate this. This multiportein complexes, thus aid in assessing the status of all the metabolites in the pathway.
4. **Mapping of Protein Modifications:** It deals with identifying how and where the proteins are modified. Many common post translational modifications govern the targeting, structure, function and turnover of proteins. Environmental chemicals, endogenous chemicals give rise to reactive electrophiles that modify proteins. The software tools for analytical studies to identify proteins and the nature of modifications come under this technique.

5. **Protein biomarkers:**
   The National Institutes of Health has defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."[
   Biomarkers of drug efficacy and toxicity are becoming a key need in the drug development process. Mass spectral-based proteomic technologies are ideally suited for the discovery of protein biomarkers in the absence of any prior knowledge of quantitative changes in protein levels.
6. **Proteogenomics**: In proteogenomics, proteomic technologies such as mass spectrometry are used for improving gene annotations. Parallel analysis of the genome and the proteome facilitates discovery of post-translational modifications and proteolytic events, especially when comparing multiple species (comparative proteogenomics).
7. **Molecular Medicine:** With the help of the information available through clinical proteomics, several drugs have been designed. This aims to discover the proteins with medical relevance to identify a potential target for pharmaceutical development, a marker(s) for disease diagnosis or staging, and risk assessment—both for medical and environmental studies.
   Proteomic technologies will play an important role in drug discovery, diagnostics and molecular medicine because of the link between genes, proteins and disease.

3. GENOMICS

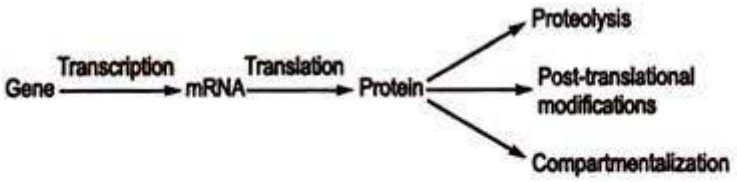
The entire genetic profile of an organism is termed as Genome. In a simpler sense, genome is the sequence of nucleic acids present in the entire set of chromosomes of the organism.

Genomics the branch of molecular biology concerned with the structure, function, evolution and mapping of genomes. Genomics is an interdisciplinary field of science within the field of molecular biology.

Many genomes of organisms have been sequenced so far, Human genome has three billion base pairs. A bacterial genome has about 60000 base pairs. DNA sequences contain the information on how to make proteins that code for structural and catalytic functions.

Genomics refers to the study of genetic information within a cell, which means the entire DNA sequence in the chromosomes of an organism. It includes gene organization, sequencing and mutations at the DNA levels as well as molecular characterization of entire genome. The term 'genomics' was introduced by T.B.Roderick in 1986.

I. Principles of genomics:



Diagrammatic representation of a gene expression showing formation of many protein isoforms from a single gene. After transcription of the gene, mRNA is alternatively spliced or edited to form a mature mRNA that is translated to the protein. Proteins can be regulated by additional mechanism of proteolysis, compartmentalization and certain other modifications

After an organism has been selected, genome projects involve three components:

1. Sequencing of DNA,
2. Assembly of that sequence to create a representation of the original chromosome
3. Annotation and analysis of that representation.

1. **DNA Sequencing:** Genome sequencing approaches fall into two broad categories, shotgun and high-throughput (or next-generation) sequencing.

a. **Shotgun sequencing:** Shotgun sequencing is a sequencing method designed for analysis of DNA sequences longer than 1000 base pairs, up to and including entire chromosomes. Since this method can only be used for fairly short sequences (100 to 1000 base pairs), longer DNA sequences must be broken into random small segments which are then sequenced to obtain reads

b. **High-throughput sequencing:** The high demand for low-cost sequencing has driven the development of high-throughput sequencing technologies that parallelize the sequencing process, producing thousands or millions of

sequences at once. In ultra-high-throughput sequencing, as many as 500,000 sequencing-by-synthesis operations may be run in parallel.

2. **Sequence assembly:** Sequence assembly refers to aligning and merging fragments of a much longer DNA sequence in order to reconstruct the original sequence. Multiple fragmented sequence reads must be assembled together on the basis of their overlapping areas.
   Assembly can be broadly categorized into two approaches:
   a. **De novo assembly**, for genomes which are not similar to any sequenced in the past.
   b. **Comparative assembly**, which uses the existing sequence of a closely related organism as a reference during assembly.
      Relative to comparative assembly, de novo assembly is computationally difficult, making it less favourable for short-read technologies.

Finished genomes are defined as having a single contiguous sequence with no ambiguities representing each replicon.

3. Genome annotation:

The DNA sequence assembly alone is of little value without additional analysis. Genome annotation is the process of attaching biological information to sequences, and consists of three main steps-

1. Identifying portions of the genome that do not code for proteins.
2. Identifying elements on the genome, a process called gene prediction.
3. Attaching biological information to these elements.

Automatic annotation tools try to perform these steps *in silico*, as opposed to manual annotation which involves human expertise and potential experimental verification. Ideally, these approaches co-exist and complement each other in the same annotation pipeline.

Traditionally, the basic level of annotation is using BLAST for finding similarities, and then annotating genomes based on homologues.

II. Types of Genomics: Genomics can be categorized mainly into 3 types.

1. Structural genomics
2. Functional genomics
3. Comparative genomics

1. **Structural genomics:** Structural genomics involves characterization of the physical nature o genome i.e. the entire nucleotide sequence of an organism. This is accomplished by genetic mapping, physical mapping and entire genome sequencing.
   The genetic profile of more than 500 organisms has been established while the nucleotide sequencing of a large number of organisms are still being carried out.
2. **Functional genomics:** Functional genomics involves analysis of genome function in any organism at various developmental stages and environmental conditions applying different techniques.

The successful completion of the Human Genome Project (HGP) has been possible using mass-scale sequencing methodology, DNA/RNA microassay,

protein chips, Transfection, Real-time PCR, Mutational analysis and mass spectrometry, all of which produce large amount of data in a short time.

3. **Comparative genomics:** Comparative genomics involves comparing the information contained in the genome from different species, such as the number of base pairs, number of genes, number of chromosomes and average gene density, introns and exons of a gene.

III. Applications of genomics: Genomics has provided applications in many fields, including medicine, biotechnology, anthropology and other social sciences.

1. **Genomic medicine:** Next-generation genomic technologies allow clinicians and biomedical researchers to drastically increase the amount of genomic data collected on large study populations.
   When combined with new informatics approaches that integrate many kinds of data with genomic data in disease research, this allows researchers to better understand the genetic bases of drug response and characterization of disease.
   It can be employed in the discovery of newer prophylactic measures and treatments.
   It can be applied for tailoring the treatment according to individual patient's need.
   It is helpful in medical research for faster development of new drugs.

2. **Synthetic biology and bioengineering:** The growth of genomic knowledge has enabled increasingly sophisticated applications of synthetic biology.
   For example, the creation of a partially synthetic species of bacterium, *Mycoplasma laboratorium*, derived from the genome of *Mycoplasma genitalium*.

3. **Conservation genomics:** Conservationists can use the information gathered by genomic sequencing in order to better evaluate genetic factors key to species conservation, such as the genetic diversity of a population or whether an individual is heterozygous for a recessive inherited genetic disorder.

4. **Epigenetics:** Epigenetics is defined as the study of inherited changes in phenotype or gene expression caused by mechanisms other than mutations in the underlying DNA sequence. Epigenetic markers include DNA methylation and modification of histone tails.

5. **Functional Genomics:** Functional genomics encompasses the research field describing the function and interaction of both proteins and genes. This area of study is a genome-wide approach that builds on our understanding of DNA structure and sequence to focus on the dynamic aspects of interactions, including gene transcription, translation, and protein-protein interactions.

   The importance of functional genomics has increased following the completion of the human genome project.

6. **Pathway Analysis:** Biological pathways include metabolic, regulatory, and signaling pathways that organize and coordinate the activities of a cell. Biological pathways often interact with one another to form biological networks. Pathway analysis is used in systems biology to build a better

picture of how the individual components of a biological system interact to create the larger functional system.

7. **Biomarker Discovery:** The National Institutes of Health (NIH) defines a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. The goal of biomarker research is to discover gene or protein markers and use them to improve diagnostic, prognostic, or therapeutic outcomes for patients, and to assist in the development of novel drug candidates.

8. **Agricultural applications:** Studying the genomics of plant and their pathogens (eg: fungi) helps in increasing the nutritional food value, improving crop yields and effectively treating the plant diseases.

9. **Animal husbandry:** Genome sequencing of farm animals such as cows, pigs and sheep are being carried out in research laboratories in the hope to widen the perspective of the biology of these organisms and improve their production and health.

10. **Environmental applications:** Solving numerous of problems relating to environment, development of new sources of energy and improvement in industrial processes can be brought about by studying and using the genomes of microbes that can survive under extreme conditions like cold, heat, pressure and radiation.

4. NANOBIOTECHNOLOGY

Nanobiotechnology is considered to be the unique fusion of biotechnology and nanotechnology and the application of nanotechnology in biological fields.

Nanotechnology (sometimes referred to as nanotech) is defined as the design, development and application of materials & devices whose least functional make up is on a nanometer scale. Generally, nanotechnology deals with developing materials, devices, or other structures possessing at least one dimension sized from 1 to 100 nanometers.

Biotechnology deals with metabolic and other physiological processes of biological subjects including microorganisms. It is the application of principles of science and technology on biological agents such as organisms, systems and processes for manufacturing and service industries.

Association of these two technologies, i.e. **nanobiotechnology** can play a vital role in developing and implementing many useful tools in the study of life.

Nanotechnology is a multidisciplinary field that currently recruits approach, technology and facility available in conventional as well as advanced avenues of engineering, physics, chemistry and biology.

I. Principles of Nanobiotechnology:

Implementation of nanotechnology in medicine and physiology means that mechanisms and devices are so technically designed that they can interact with sub-cellular (i.e. molecular) levels of the body with a high degree of specificity. Thus therapeutic efficacy can be achieved to maximum with minimal side effects by means of the targeted cell or tissue-specific clinical intervention.

Most of the scientific concepts in bionanotechnology are derived from other fields. Biochemical principles that are used to understand the material properties of biological systems are central in bionanotechnology because those same principles are to be used to create new technologies.

Material properties and applications studied in bionanoscience include

- Mechanical properties (e.g. deformation, adhesion, failure),
- Electrical/electronic properties (e.g. electromechanical stimulation, capacitors, energy storage/batteries),
- Optical properties (e.g. absorption, luminescence, photochemistry),
- Thermal properties (e.g. thermomutability, thermal management),
- Biological properties where the interactions of cells with nanomaterials, molecular flaws/defects, biosensing, biological mechanisms were studied.
- Nanoscience of disease (e.g. genetic disease, cancer, organ/tissue failure

- DNA computing
- Agricultural phenomenon for target delivery of pesticides, hormones and fertilizers.

II. Applications of Nanobiotechnology: Nanotechnology application to biotechnology leaves no field untouched by its groundbreaking scientific innovations for human wellness. Some of these applications are given below.

1. Nanomedicine

A number of clinical applications of nanobiotechnology, such as disease diagnosis, target-specific drug delivery, and molecular imaging are being laboriously investigated at present.

The pathophysiological conditions and anatomical changes of diseased or inflamed tissues can potentially trigger a great deal of scopes for the development of various targeted nanotechnological products. This development is like to be advantageous in the following ways:

- Drug targeting can be achieved by taking advantage of the distinct pathophysiological features of diseased tissues
- Various nanoproducts can be accumulated at higher concentrations than normal drugs.
- increased vascular permeability coupled with an impaired lymphatic drainage in tumors improve the effect of the nanosystems in the tumors or inflamed tissues through better transmission and retention
- Nanosystems have capacity of selective localization in inflammed tissues.
- Nanoparticles can be effectively used to deliver/transport relevant drugs to the brain overcoming the presence of blood–brain barrier (meninges).
- Drug loading onto nanoparticles modifies cell and tissue distribution and leads to a more selective delivery of biologically active compounds to enhance drug efficacy and reduces drug toxicity.

2. **Protein chips:** Proteins play the central role in establishing the biological phenotype of organisms in healthy and diseased states and are more indicative of functionality. Hence, proteomics is important in disease diagnostics and pharmaceutics, where drugs can be developed to alter signaling pathways. Protein chips can be treated with chemical groups, or small modular protein components, that can specifically bind to proteins containing a certain structural or biochemical moiety.
3. **Sparse cell detection:** Sparse cells are both rare and physiologically distinct from their surrounding cells in normal physiological conditions (e.g. cancer cells, lymphocytes, fetal cells and HIV-infected T cells). They are significant in the detection and diagnosis of various genetic defects. However, it is a challenge to identify and subsequently isolate

these sparse cells. Nanobiotechnology presents new tools and effective ways to identify these cells.

4. **Drug Delivery:** Nanoparticles as therapeutics can be delivered to targeted sites, including locations that cannot be easily reached by standard drugs and in the effective design of specific release drug delivery systems.

   Many agents, which cannot be administered orally due to their poor bioavailability, will now have scope of use in therapy with the help of nanotechnology.

   Nano-formulations offer protection for agents vulnerable to degradation or denaturation when exposed to extreme pH, and also prolong half-life of a drug by expanding retention of the formulation through bioadhesion

5. **Delivery of antigens for vaccination:** Recent advances in encapsulation and development of suitable animal models have demonstrated that microparticles and nanoparticles are capable of enhancing immunization.

6. **Gene therapy:**

   Current gene therapy systems suffer from the inherent difficulties of effective pharmaceutical processing and development, and the chance of reversion of an engineered mutant to the wild type. Potential immunogenicity of viral vectors involved in gene delivery is also problematic.

   This problem can be solved by using nanotechnological tools and nanoparticle-based nonviral vectors (usully 50-500 nm in size) to transport plasmid DNA and are beneficial in repairing or replacing impaired genes in human.

7. **Biomolecular Engineering:** The expense and time involved in traditional biomolecule designing limit the availability of bioactive molecules. Nanoscale assembly and synthesis techniques provide an alternative to traditional methods. Improvements can be achieved due to the ability to carry out chemical and biological reactions on solid substrates, rather than through the traditional solution based processes.

8. **Biopharmaceuticals applications:** Nanobiotechnology can develop drugs for diseases that conventional pharmaceuticals cannot target. Nanobiotechnology brings the ability to physically manipulate targets, molecules and atoms on solid substrates by tethering them to biomembranes and controlling where and when chemical reactions take place, in a fast process that requires few materials (reagents and solutions). This advance will reduce drug discovery costs, will provide a large diversity of compounds, and will facilitate the development of highly specific drugs.

9. **Nanotechnology in cardiac therapy**: Nanotechnology is currently offering promising tools for applications in modern cardiovascular science to explore existing frontiers at the cellular level and treat challenging cardiovascular diseases more effectively.

    These tools can be applied in diagnosis, imaging and tissue engineering. Miniaturized nanoscale sensors like quantum dots (QDs), nanocrystals, and nanobarcodes are capable of sensing and monitoring complex immune signals in response to cardiac or inflammatory events.

    Nanotechnology can also help detect and describe clinically-significant specific mechanisms implicated in cardiac disorders.

    In addition, it is useful in designing atomic-scale machines that can be incorporated into biological systems at the molecular level.

10. **Nanotechnology in dental care:** The role of nanodentistry by means of the use of nanomaterials, biotechnology and nanorobotics will ensure better oral health. Reconstructive dental nanorobots could be used in selective and precise occlusion of specific tubules within minutes, and this will facilitate quick and permanent recovery. Covalently-bonded artificial materials like sapphire may replace upper enamel layer to boost the appearance and durability of teeth.

11. **Nanotechnology in orthopaedics:** Nanomaterials sized between 1 and 100 nm have role to play as new and functional constituents of bones being also made up of nanosized organic and mineral phases. Nanomaterials, nanopolymers, carbon nanofibers, nanotubes, and ceramic nanocomposites may help with more efficient deposition of calcium-containing minerals on implants.

12. **Agriculture:**

In the agriculture industry, engineered nanoparticles have been serving as nano carriers, containing herbicides, chemicals, or genes, which target particular plant parts to release their content.

5. GENE THERAPY

Gene therapy is an experimental technique that uses genes to treat or prevent diseases and genetic disorders by the therapeutic introduction of normal genes into cells in place of missing or defective ones.

All medical procedures that introduce alterations to a patient's genetic makeup can be considered gene therapy. Bone marrow transplantation and organ transplants in general have been found to introduce foreign DNA into patients.

Approaches for gene therapy:

Following early advances in genetic engineering of bacteria, cells, and small animals, scientists started considering how to apply it to medicine.

Researchers are testing several approaches to gene therapy, including:

- Replacing a mutated gene that causes disease with a healthy copy of the gene.
- Inactivating, or "knocking out," a mutated gene that is functioning improperly.
- Introducing a new gene into the body to help fight a disease.

Two main approaches were considered in gene therapy

1. Replacing genes
2. Disrupting defective genes.

Both are designed to introduce genetic material into cells to compensate for abnormal genes or to make a beneficial protein. If a mutated gene causes a necessary protein to be faulty or missing, gene therapy may be able to introduce a normal copy of the gene to restore the function of the protein.

A gene that is inserted directly into a cell usually does not function. Instead, a carrier called a vector is genetically engineered to deliver the gene. Certain viruses are often used as vectors because they can deliver the new gene by infecting the cell. The viruses are modified so they can't cause disease when used in people. Some types of virus, such as retroviruses, integrate their genetic material (including the new gene) into a chromosome in the human cell. Other viruses, such as adenoviruses, introduce their DNA into the nucleus of the cell, but the DNA is not integrated into a chromosome.

The vector can be injected or given intravenously (by IV) directly into a specific tissue in the body, where it is taken up by individual cells. Alternately, a sample of the patient's cells can be removed and exposed to the vector in a laboratory setting. The cells containing the vector are then returned to the patient. If the treatment is successful, the new gene delivered by the vector will make a functioning protein.

Researchers must overcome many technical challenges before gene therapy will be a practical approach to treating disease. For example, scientists must find better

ways to deliver genes and target them to particular cells. They must also ensure that new genes are precisely controlled by the body.

A new gene is injected into an adenovirus vector, which is used to introduce the modified DNA into a human cell. If the treatment is successful, the new gene will make a functional protein.

CLASSIFICATION OF GENE THERAPY: Gene therapy may be classified into two types-

- ✓ Somatic cell gene therapy (SCGT)
- ✓ Germline gene therapy (GGT)

1. **Somatic cell gene therapy (SCGT):** In somatic cell gene therapy, the therapeutic genes are transferred into any cell other than a gamete (haploid cell that fuses with another haploid cell during fertilization (conception) in organisms that sexually reproduce), germ cell, gametocyte (spermatocytes and oocytes), or undifferentiated stem cell.

   Any such modifications affect the individual patient only, and are not inherited by offspring i.e. it repair or replace defective gene in germ line cell. Modified gene would be inherited. The therapeutic DNA (either integrated in the genome or as an external episome or plasmid) is used to treat disease.

2. **Germline gene therapy (GGT):** In germline gene therapy, germ cells (sperm or egg cells) are modified by the introduction of functional genes into their genomes. Modifying a germ cell causes all the organism's cells to contain the modified gene. The change is therefore heritable and passed on to later generations.

VECTORS IN GENE THERAPY: Vectors can be used to inject the specific gene into the host cells.

Requirements for Vector

The ideal requirements for vectors are

- ➢ It should not be identified by immune system (non-immunologic)
- ➢ Should be stable and easy to reproduce
- ➢ Should have longevity of expression
- ➢ Should have high efficiency (100% cells transfected)
- ➢ High specificity and low toxicity
- ➢ It should be able to protect and deliver DNA across the cell membrane into the nucleus. It should be able to target gene delivery to specific cells
- ➢ It should be easy to be produced in large amounts and be inexpensive

Currently no single vector type will meet all needs for all tissues, that is different vectors will be needed for different clinical applications.

The delivery of DNA into cells can be accomplished by multiple methods.

Classes of vectors: The two major classes are ***recombinant viruses*** **(**sometimes called biological nanoparticles or viral vectors) and ***non-viral methods*** (naked DNA or DNA complexes).

1. **Viruses:** In order to replicate, viruses introduce their genetic material into the host cell, tricking the host's cellular machinery using it as blueprints for viral proteins. Retroviruses go a stage further by having their genetic material copied into the genome of the host cell by substituting a virus's genetic material with therapeutic DNA.

A number of viruses have been used for human gene therapy, including retroviruses, adenoviruses, herpes simplex, vaccinia, and adeno-associated virus.

2. **Non-viral methods:** Non-viral methods present certain advantages over viral methods, such as large scale production and low host immunogenicity. However, non-viral methods initially produced lower levels of transfection and gene expression, and thus lower therapeutic efficacy.

Methods for non-viral gene therapy include the injection of naked DNA, electroporation, the gene gun, sonoporation, magnetofection, the use of oligonucleotides, lipoplexes, dendrimers, and inorganic nanoparticles.

APPLICATIONS OF GENE THERAPY: Gene therapy has many therapeutic and cosemetic applications but most of them are still under research. Though some of them are proven to be successful they do have some ethical issues.

The following are the some of the applications of gene therapy.

1. Bone repair: The bone morphogenic proteins (BMPs) enable skeletal tissue formation during embryogenesis, growth, adulthood, and healing. It will be possible to directly deliver the BMP gene in vivo to tissues via an adeno viral vector to heal bone defects The advantage of an ex vivo gene transfer approach is that specific cells like bone marrow cells or stem cells can be selected as the cellular delivery vehicle for specific clinical problems. In addition, ex vivo strategies have a high efficiency of cell transduction.

2. Tissue engineering*:* Gene constructs, such as plasmid DNA or a viral particle are physically entrapped within a matrix. When this matrix/scaffold is implanted into the tissue defects, host cells migrate into the implant, take up the gene construct and start producing the encoded protein.

3. Pain Management: Managing or eliminating pain is a major part of dental practice. The use of gene transfer technology offers a potentially novel approach to manipulate specific, localized biochemical pathways involved in pain generation. Gene transfer may be particularly useful for managing chronic and intractable pain.

4. DNA Vaccination: By directly delivering DNA in a plasmid rather than the traditional administration of a purified protein or an attenuated microbe, the ability to induce an immune response to a protein antigen can be achieved.

5. Transplanting keratinocyte: Gene therapy can be applied for transplanting keratinocytes sheets for burn patients and to treat various skin diseases. It is reversible because genetically modified tissue can be excised. Cultured oral keratinocytes have been grafted to oral surgical defects. They persist at these sites and exhibit normal epithelial morphology.

6. Treatment of cancer: The general strategy in cancer treatment is to express a gene product that will result in cancer cell death. It can be achieved by

- Addition of a tumor-suppressor gene (gene addition therapy).
- Deletion of a defective tumor gene (gene excision therapy).
- Down-regulation of the expression of genes that stimulate tumor growth.
- Enhancement of immune surveillance (immunotherapy).
- Activation of pro-drugs that have a chemotherapeutic effect and cause toxicity only to tumor cells ("suicide" gene therapy);
- Introduction of genes to inhibit tumor angiogenesis.
- "Cancer vaccination" with genes for tumor antigens.

7. Fertility: Gene Therapy techniques have the potential to provide alternative treatments for those with infertility. In mice, it has proven that fertility can be restored by using the gene therapy method, Spermatogenical stem cells from another organism were transplanted into the testes of an infertile male mouse. The stem cells re-established spermatogenesis and fertility.

8. Human genetic engineering: Genetic engineering could be used to change physical appearance, metabolism, and even improve physical capabilities and mental faculties such as memory and intelligence.

9. Gene doping: Athletes might adopt gene therapy technologies to improve their performance. Gene doping is not known to occur, but multiple gene therapies may have such effects. However this has ethical issues.

In the future, gene therapy may allow doctors to treat a disorder by inserting a gene into a patient's cells instead of using drugs or surgery.

DIFFICULTIES IN GENE THERAPY: Although gene therapy is a promising treatment option for a number of diseases (including inherited disorders, some types of cancer, and certain viral infections), the technique remains risky and is still under study to make sure that it will be safe and effective. Gene therapy is currently being tested only for diseases that have no other cures.

The difficulties include

- Difficulty to deliver genes in some sites like lung cells
- Genes might integrate at sites where it can affect the functioning of another gene
- Vectors may be recognized as —foreign by immune system triggering immune response
- Viral vector may cause toxicity, inflammatory response and might recover their ability to cause disease
- Multigene disorders are difficult to treat by gene therapy
- Gene therapy is expensive.

Introduction to Antigen-Antibody Reactions

Antigen-antibody reaction is the basis of humoral immunity or antibody mediated immune response.

The interactions between antigens and antibodies are known as ***antigen–antibody reactions***. The reactions are highly specific, and an antigen reacts only with antibodies produced by itself or with closely related antigens. Antibodies recognize molecular shapes (epitopes) on antigens. Generally, the better the fit of the epitope (in terms of geometry and chemical character) to the antibody combining site, the more favourable the interactions that will be formed between the antibody and antigen and the higher the affinity of the antibody for antigen. The affinity of the antibody for the antigen is one of the most important factors in determining antibody efficacy *in vivo*.

The antigen- antibody interaction is bimolecular irreversible association between antigen and antibody. The association between antigen and antibody includes various non-covalent interactions between epitope (antigenic determinant) and variable region (V_H/V_L) domain of antibody.

Chemical Bonds Responsible for the Antigen–Antibody Reaction

The interaction between the Ab-binding site and the epitope involves exclusively non-covalent bonds, in a similar manner to that in which proteins bind to their cellular receptors, or enzymes bind to their substrates. The binding is reversible and can be prevented or dissociated by high ionic strength or extreme pH. The following intermolecular forces are involved in Ag–Ab binding:

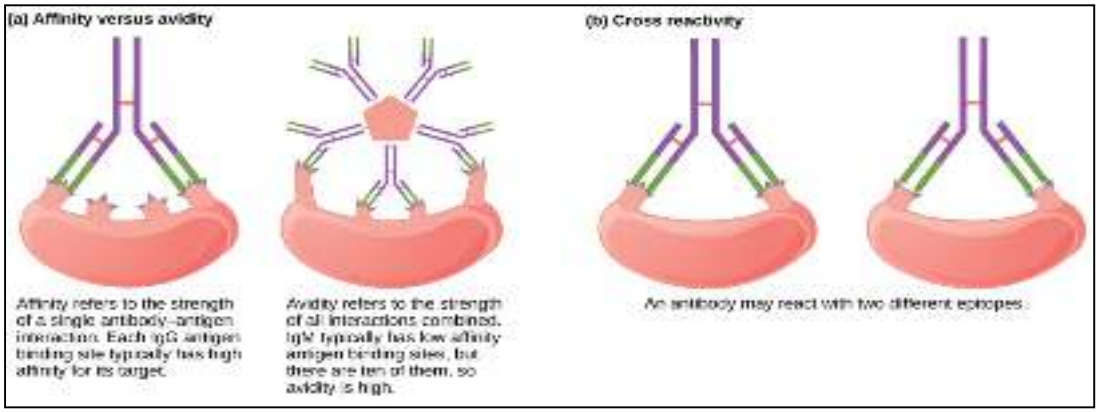
1. **Electrostatic bonds:** This result from the attraction between oppositely charged ionic groups of two protein side chains; for example, an ionized amino group (NH_4^+) on a lysine in the Ab, and an ionized carboxyl group (COO^-) on an aspartate residue in the Ag.
2. **Hydrogen bonding**: When the Ag and Ab are in very close proximity, relatively weak hydrogen bonds can be formed between hydrophilic groups (e.g., OH and C=O, NH and C=O, and NH and OH groups).
3. **Hydrophobic interactions**: Hydrophobic groups, such as the side chains of valine, leucine, and phenylalanine, tend to associate due to Vander Waals bonding and coalesce in an aqueous environment, excluding water molecules from their surroundings. As a consequence, the distance between them decreases, enhancing the energies of attraction involved. This type of interaction is estimated to contribute up to 50% of the total strength of the Ag–Ab bond.
4. **Vander Waals bonds:** These forces depend upon interactions between the "electron clouds" that surround the Ag and Ab molecules. The interaction has been compared to that which might exist between alternating dipoles in two molecules, alternating in such

a way that, at any given moment, oppositely oriented dipoles will be present in closely apposed areas of the Ag and Ab molecules.

Each of these non-covalent interactions operates over very short distance (generally about 1 Å) so, Ag-Ab interactions depends on very close fit between antigen and antibody.

Strength of Ag-Ab interactions

1. **Affinity**
   - Combined strength of total non-covalent interactions between single Ag- binding site of Ab and single epitope is affinity of Ab for that epitope.
   - Low affinity Ab: Bind Ag weakly and dissociates readily.
   - High affinity Ab: Bind Ag tightly and remain bound longer.
2. **Avidity**
   - Strength of multiple interactions between multivalent Ab and Ag is avidity. Avidity is better measure of binding capacity of antibody than affinity. High avidity can compensate low affinity.
3. **Cross reactivity**
   - Antibody elicited by one Ag can cross react with unrelated Ag if they share identical epitope or have similar chemical properties.



Types of Ag-Ab reactions – Stages

In vitro

1. Agglutination
2. Precipitation
3. Neutralization
4. Complement Fixation

In vivo

1. Agglutination
2. Precipitation
3. Complement Fixation
4. Neutralization
5. Enzyme Linked Immunosorbent Assay (ELISA)
6. RadioImmuno Assay (RIA)
7. Western Blotting
8. Fluorescent ag-ab technique

Antigen and Antibody react with each other in three stages: 3 stages

Primary stage	**Secondary stage**	**Tertiary stage**
Combination of antigen + antibody	1. Agglutination	1. Opsonization
	2. Precipitation	2. Lysis
	3. Complement activation	
	4. Neutralization	
	5. Blocking of antigen sites	

1. Primary Stage

In the primary stage, there is a combination of antigen and antibody that gives rise to antigen and antibody complex formation (Ag + Ab).

Primary stage is the initial interaction between antigen and antibody. It is rapid and **reversible,** but without any visible effects. The ionic bonds, hydrogen bonds, van der Waals forces, and hydrophobic interactions are the weaker intermolecular forces that bind antigen and antibodies together in this primary stage.

Covalent binding, which is a stronger intermolecular force between antigen and antibody, however, does not occur in this stage.

2. Secondary stage

Secondary stage is an **irreversible interaction** between anti-gen and antibody, with **visible effects,** such as agglutination, precipitation, neutralization, complement fixation, and immobilization of motile organisms. The binding between antigen and antibody during this stage occurs by covalent binding.

A single antibody is capable of causing different types of antigen–antibody reactions, and a single antigen is capable of inducing production of different classes of immunoglobulins, which differ in their biological properties.

The results of agglutination, precipitation, neutralization, and other tests are usually expressed as a titer. ***Titer*** is defined as the highest dilution of serum that gives a positive reaction in test. Higher titer means greater level of antibodies in serum. For example, a serum with a titer of 1/128 contains more antibodies than a serum with a titer of 1/8.

3. Tertiary stage

Some antigen-antibody reactions occurring in vivo initiate chain reactions that lead to neutralization or destruction / opsonizaton / lysis of injurious antigens, or to tissue damage. These are the tertiary reactions and include humoral immunity against infectious disease as well as clinical allergy and other immunological diseases.

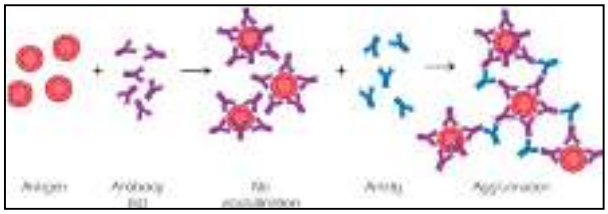
1. Agglutination

- ✓ Agglutination is an antigen –antibody reaction where the antibody of serum causes the cellular antigen to adhere to one another to form clumps.
- ✓ It is the clumping of a particular antigen and its antibody.
- ✓ The antibody that cause agglutination are called agglutinins and particulate antigens aggregated are called agglutinogens.
- ✓ The particulate antigen includes bacterial, viruses, RBC, platelets lymphocytes, etc.
- ✓ When red blood called are agglutinated, the reaction is called Heamagglutination.
- ✓ When bacterial cells are agglutinated, the agglutination is called Bacterial Agglutination.

Simple, inexpensive, but sensitive!

Several types exist:

a) Hemagglutination of RBC's
b) Bacterial Agglutination
c) Passive Agglutination
d) Agglutination Inhibition



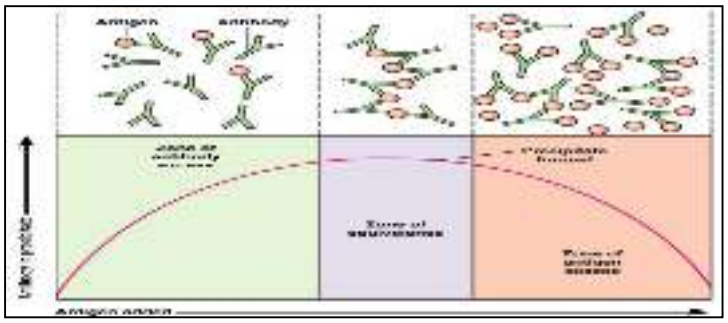
Application of Agglutination reaction:

1. Cross-matching and grouping of blood.
2. Identification of Bacteria. Ex: Serotyping of *Vibrio cholera*, Serotyping of *Salmonella Typhi* and Paratyphi.
3. Serological diagnosis of various diseases. Ex: Rapid plasma regains (RPR) test for Syphilis, Antistreptolysin O (ASO) test for rheumatic fever.
4. Detection of unknown antigen in various clinical specimens. E.g. detection of antigen of *Salmonella typhi* in the urine.

2. Precipitation

It is a type of antigen-antibody reaction, in which the antigen occurs in a soluble form. When a soluble antigen reacts with its specific antibody, at an optimum temperature and pH in the presence of electrolyte antigen-antibody complex forms insoluble precipitate. This reaction is called a precipitation reaction. A lattice is formed between the antigens and antibodies; in certain cases, it is visible as an insoluble precipitate. Antibodies that aggregate soluble antigens are called precipitins.

Antigen (Soluble)	+	Antibody -------- Precipitation (Soluble)
Antigen (Insoluble)	+	Antibody -------- Flocculation / Agglutination (Soluble)



Precipitation curve

Steps 1:

- ✓ Same proportion of Ag-Ab interact with each other by weak intramolecular forces
- ✓ Not visible
- ✓ No precipitate / lattice

Steps 2:

- ✓ Ag-Ab complement with each other and trying to cross link
- ✓ Not visible

Steps 3:

- ✓ Cross linking takes place
- ✓ Lattice formed
- ✓ Visible

Precipitation Reactions in Gels Yield Visible Precipitin Lines: Immune precipitates can form not only in solution but also in an agar matrix. When antigen and antibody diffuse toward one another in agar, or when antibody is incorporated into the agar and antigen diffuses into the antibody-containing matrix, a visible line of precipitation will form.

As in a precipitation reaction in fluid, visible precipitation occurs in the region of equivalence, whereas no visible precipitate forms in regions of antibody or antigen excess. Two types of immunodiffusion reactions can be used to determine relative concentrations of antibodies or antigens, to compare antigens, or to determine the relative purity of an antigen preparation.

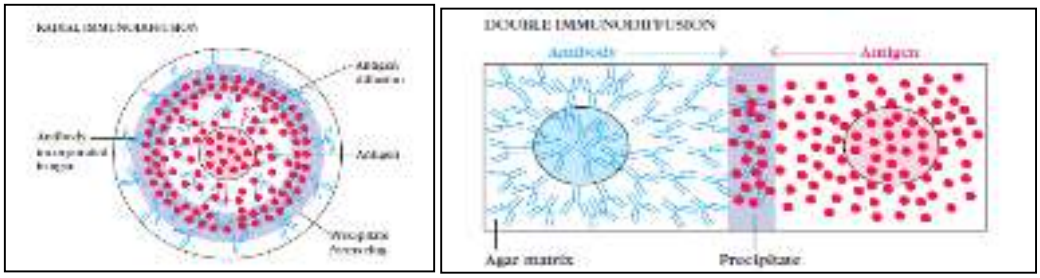
They are:

1. Radial immunodiffusion (the Mancini method)
2. Double immunodiffusion (the Ouchterlony method)

} both are carried out in a semisolid medium such as agar.

1. Radial immunodiffusion (the Mancini method)

In radial immunodiffusion, an antigen sample is placed in a well and allowed to diffuse into agar containing a suitable dilution of an antiserum. As the antigen diffuses into the agar, the region of equivalence is established and a ring of precipitation, a precipitin ring, forms around the well (Figure 6-5, upper panel). The area of the precipitin ring is proportional to the concentration of antigen. By comparing the area of the precipitin ring with a standard curve (obtained by measuring the precipitin areas of known concentrations of the antigen), the concentration of the antigen sample can be determined.



Radial immunodiffusion **Double immunodiffusion**

2. Double immunodiffusion (the Ouchterlony method)

In the Ouchterlony method, both antigen and antibody diffuse radially from wells toward each other, thereby establishing a concentration gradient. As equivalence is reached, a visible line of precipitation, a precipitin line, forms

Diagrammatic representation of radial immunodiffusion (Mancini method) & double immunodiffusion (Ochterlony method) in a gel. In both cases, large insoluble complexes form in the agar in the zone of equivalence, visible as line of precipitation (purple regions). Only the antigen (red) diffuses in radial immunodiffusion, where as both the antibody (blue) and antigen (red) diffuse in double immunodiffusion

Advantages

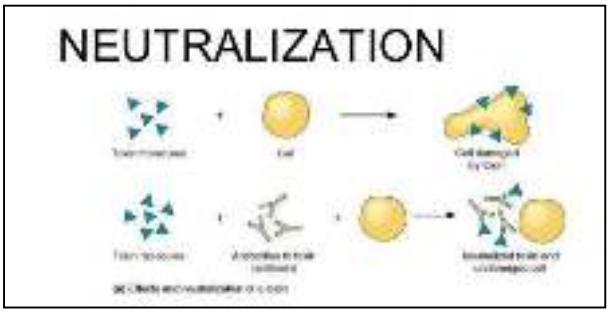
- Fairly sensitive
- High specificity

Disadvantages

- Time consuming Time consuming
- Some costly instruments are required
- High technical skill required

3. Neutralization

Bacterial exotoxins are capable of producing neutralizing antibodies (antitoxins) which play protective role in diseases such as diphtheria and tetanus.
Toxin – antitoxin neutralization can be measured in vivo and in vitro.



Neutralization is an antigen–antibody reaction in which the biological effects of viruses and toxins are neutralized by homologous antibodies known as neutralizing antibodies.

These tests are broadly of two types:

(a) Virus neutralizationν tests

(b) Toxin neutralization tests

Neutralizing antibodies block or distort the antigenν sufficiently, so that pathogen fails to exert its biological activity. Neutralization reactions can occur in vitro or in vivo.ν Laboratory animals or tissue culture cells are used asν "indicator systems" in neutralization tests. The toxin or virus to be assayed should have known effects on the indicator system which is neutralized by antibodies.

Toxin neutralization tests

Toxin neutralization tests are based on the principle that biological action of toxin is neutralized on reacting with specific neutralizing antibodies called antitoxins.

Examples of toxin neutralization tests include:

In vivo:
(a) Schick test to demonstrate immunity againstν diphtheria and

(b) Clostridium welchii toxin neutralization test in guineaν pig or mice.

In vitro:

(a) Antistreptolysin O test

(b) Nagler reaction used for rapid detection of *Clostridium welchii*

Virus Neutralization Test

Neutralization of viruses by their specific antibodies are calledv virus neutralization tests. Inoculation of viruses in cell cultures, eggs, and animalsv results in the replication and growth of viruses. When virus specific neutralizing antibodies are injected into these systems, replication and growth of viruses is inhibited. This forms the basis of virus neutralization test. Neutralizing antibodies may interfere with virion binding tov receptors, block uptake into cells, prevent uncoating of the genomes in endosomes, or cause aggregation of virus particles. Neutralizing antibody is directed against the surface proteinsv of the virus. Antibodies formed against internal components of the virus do not neutralize the infectivity of the virus; rather such non-neutralizing antibodies may enhance their infectivity

4. Complement Fixation Test- Steps, Advantages and Disadvantages

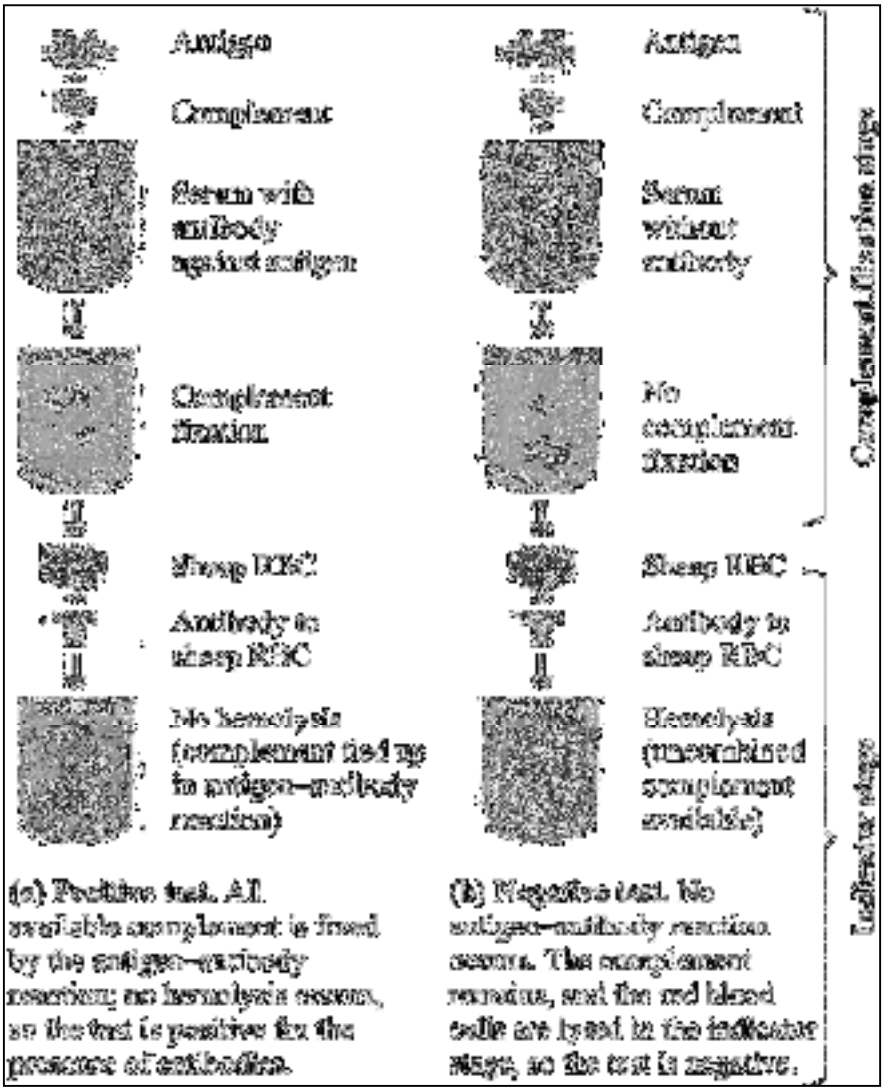
It is a classic method for demonstrating the presence of antibody in patient serum. It is based on the principle that antigen-antibody complex fixes the complement. As coupling of complement has no visible effects or changes, it is necessary to use an indicator system consisting of sheep RBC and coated with anti-sheep RBC antibody. Complement lyses antibody coated RBC.

Steps of Complement Fixation Test

Step 1: A known antigen and inactivated patient's serum are incubated with a standardized, limited amount of complement. If the serum contains specific, complement activating antibody the complement will be activated or fixed by the antigen-antibody complex. However, if there is no antibody in the patient's serum, there will be no formation of antigen-antibody complex, and therefore complement will not be fixed. But will remain free.

Step 2: The second step detects whether complement has been utilized in the first step or not. This is done by adding the indicator system. If the complement is fixed in the first step owing to the presence of antibody there will be no complement left to fix to the indicator system. However, if there is antibody in the patient's serum, there will be no antigen-antibody complex, and therefore, complement will be present free or unfixed in the mixture. This unfixed complement will now react with the antibody- coated sheep red blood cells to bring about their lysis.

Thus, no lysis of sheep red blood cells (positive CFT) indicates the presence of antibody in the test serum, while lysis of sheep red blood cells (Negative CFT) indicates the absence of antibody in the serum.



Advantages of Complement Fixation Test

1. Ability to screen against a large number of viral and bacterial infections at the same time.
2. Economical.

Disadvantages of Complement Fixation Test

1. Not sensitive – cannot be used for immunity screening.
2. Time-consuming.
3. Often non-specific e.g. cross-reactivity between Herpes Simplex Virus and Voricella Zoster Virus.

https://www.youtube.com/watch?v=EfLJGR0U2gQ

5. ELISA- Principle, Types and Applications

Enzyme-linked Immunosorbent Assay (shortened as ELISA) is used to identify peptides, proteins, antibodies and hormones. Also, called as enzyme immunoassay (EIA), ELISA finds use in the fields of biotechnology and medicine as a diagnostic tool. Mainly, antibodies and

colour changes are used to identify target substances. Also, ELISAs are useful in measuring antigen and antibody concentration.

Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) are both widely used as diagnostic tools in medicine and as quality control measures in various industries; they are also used as analytical tools in biomedical research for the detection and quantification of specific antigens or antibodies in a given sample

How does ELISA work?

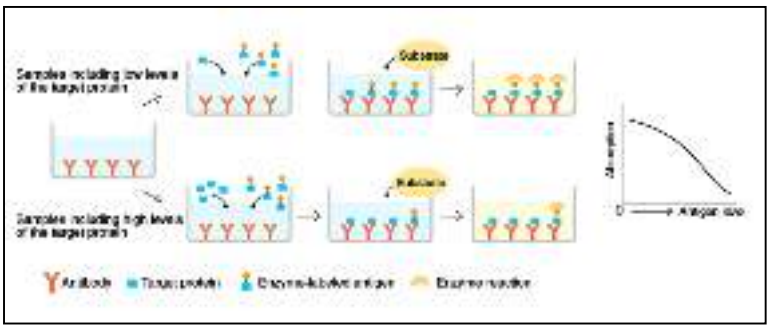
ELISA is based on specific antigen-antibody reaction and usually involves immobilizing antibodies or antigens to a 96-well or 384-well plate. The basic steps of ELISA:

ELISAs are typically performed in 96-well polystyrene plates. The serum is incubated in a well, and each well contains a different serum.

1. Immobilization of the target proteins/antigens on the surface of a microplate
2. Washing unbound/excess proteins/antigens from the plate
3. Adding a labelled antibody which will subsequently bind the target antigen/protein present in the plate
4. Washing unbound (excess) antibodies off the plate
5. Adding enzyme-specific substrates that will react with the enzyme and produce a colored product, which can be measured colorimetrically using a microplate reader.

Common enzymes used in ELISA: Horse radish peroxidase (HRP) or alkaline phosphatase
Common substrates include: Tetramethylbenzidine (TMB) and 2, 2′-azino-bis-3-Ethylbenzthiazoline-6-sulphonic acid (*ABTS*)

Duplicate or triplicate sampling is generally preferred and different concentrations of the sample are used to ensure biologically acceptable range of detection.

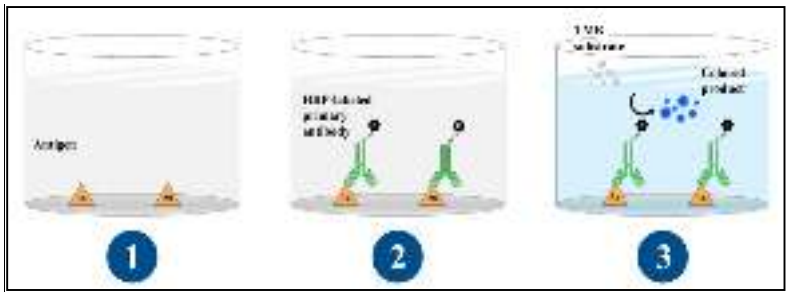


ELISA – Types:

1. Direct ELISA
2. Indirect ELISA
3. Sandwich ELISA
4. Competitive ELISA

1. **Direct ELISA-** *Antigen screening; detect soluble antigen*

The detection via direct ELISA procedure is much faster as compared to other ELISA techniques due to limited steps followed. This technique allows for coating of target protein / antigen directly to wells of microtitre plate which is followed by the addition of enzyme labelled primary antibody that detects the antigen that is complementary. The assay is beneficial as it is minimally error prone due to the implementation of a lesser number of steps requiring only a handful of reagents. There *is no requisite of cross-reacting secondary antibody in this technique.* But this technique poses a few disadvantages with regards to its specificity.



Advantages

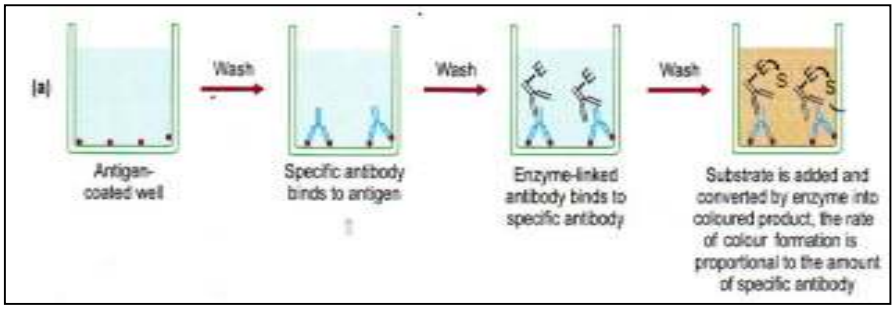
- ✓ Fast and minimal steps involved in the procedure.
- ✓ Minimum precursor requirement makes it less error prone.

Disadvantages

- ✓ The immobilization of the antigen is not specific due to which background related issues are seen.
- ✓ Offers less flexibility in terms of primary antibody.
- ✓ The absence of signal amplification reduces sensitivity.

2. Indirect ELISA- *Antibody screening; epitope mapping*

Antibody can be detected or quantitatively determined by indirect ELISA. In this technique, antigen is coated on the microtiter well. Serum or some other sample containing primary antibody is added to the microtiter well and allowed to react with the coated antigen. Any free primary antibody is washed away and the bound antibody to the antigen is detected by adding an enzyme conjugated secondary antibody that binds to the primary antibody. Unbound secondary antibody is then washed away and a specific substrate for the enzyme is added. Enzyme hydrolyzes the substrate to form colored products. The amount of colored end product is measured by spectrophotometric plate readers that can measure the absorbance of all the wells of 96-well plate.



Advantages

- ✓ Offers high sensitivity and flexibility as the number of secondary antibodies can bind to a primary antibody and one type of secondary antibody can label different primary antibodies
- ✓ It is cheaper as there is a requirement of fewer labeled antibodies.

Disadvantages

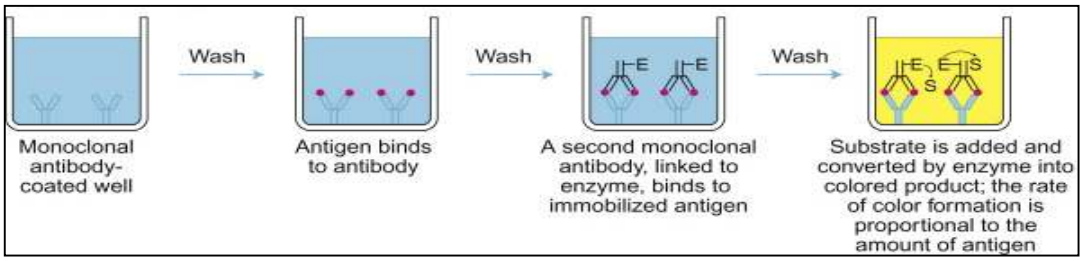
- ✓ Higher signal to noise ratio.
- ✓ Time consuming and extra labour required.

3. Sandwich ELISA

An antibody to a target protein is immobilized on the surface of microplate wells and incubated first with the target protein and then with another target protein-specific antibody, which is labeled with an enzyme. After washing, the activity of the microplate well-bound enzyme is measured. The immobilized antibody (orange) and the enzyme-labeled antibody (green) must recognize different epitopes of the target protein.

(OR)

In this system, the target antigen is detected via anchoring between two antibodies, which recognize different epitopes, or the so-called sandwich system. Sandwich ELISA starts from the immobilization of an antibody, called a capture antibody, on the microtiter plate. After blocking the plate surface to avoid non-specific adsorption of other proteins, the antigen in the sample is allowed to react with the immobilized capture antibody, and the antigen bound to the capture antibody is then sandwiched with an enzyme-labeled antibody for colour development. This direct system can be modified to the indirect system by using primary and enzyme-labeled secondary antibodies. The signal increases with increasing amount of antigen. As two antibodies containing different epitopes are required against the target antigen, sandwich ELISA is generally suitable for measuring macromolecules with some exceptions.



Advantages

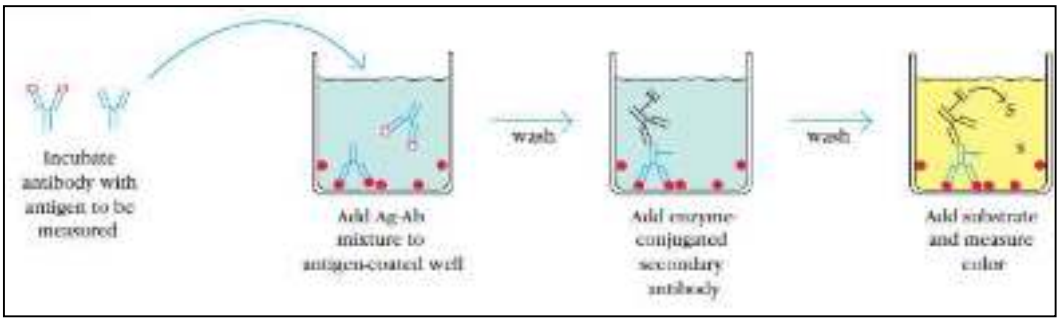
- ✓ Offers high sensitivity compared to direct or indirect ELISA
- ✓ Introduces highly specific reaction due to the involvement of two antibodies for antigen detection.
- ✓ Both direct and indirect technique is implemented in detection.

Disadvantages

- ✓ Optimization in terms of antibody becomes problematic due to cross-reactivity issues.
- ✓ For recognition of a specific epitope, only monoclonal antibodies can be applied as matched pairs.
- ✓ To procure monoclonal antibodies it is a tedious process in case of matched pairs and are more expensive than polyclonal antibodies.

5. Competition/Inhibition ELISA

In this procedure, a reference antigen is immobilized on the plate surface and biological sample pre-incubated with a specific amount of labeled antibody is added to the plate. The amount of antigen present in the sample will determine the amount of unbound or free antibodies available for binding the reference antigen in the plate.



Advantages

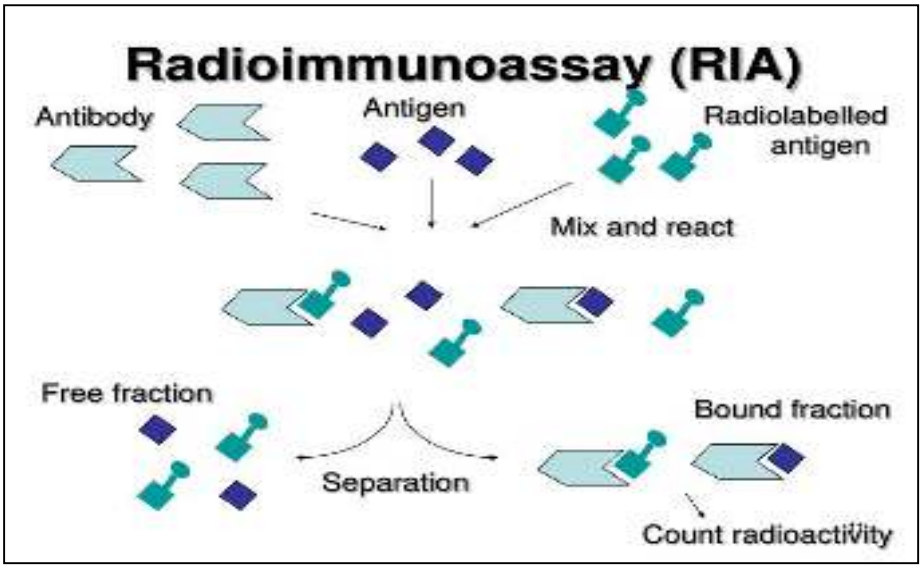
- ✓ Negligible sample processing is required and can be applicable to crude samples
- ✓ Less sensitive to experimental errors.
- ✓ Good reproducibility and flexibility.
- ✓ The main advantage of competition ELISA is its high sensitivity to compositional differences in complex antigen mixtures, even when the specific detecting antibody is present in relatively small amounts

Disadvantages

- ✓ Basic ELISA limitations apply

6. Radioimmunoassay

Radioimmunoassay (RIA) is is an *in vitro* assay and based on the principle of all immunoassays which is the recognition of an antigen present in a sample by antibodies directed against this antigen. The principle of radioimmunoassays is very similar to that of competitive ELISA and allows quantification of small molecules, peptides and proteins in biological samples.



Requirements of RIA Radioimmunoassay involves three components:
Pure antigen, radiolabeled antigen, and antiserum (antibody). In addition a separation technique is essential to estimate the distribution of radioactivity in the free and bound fractions. The sensitivity of an assay depends to a large extent on the quality of these components and choice of a suitable separation technique.

Radioactivity:
Radioisotopes are one of the main factors for immunodiagnostic technology. Usually, Iodine isotope 125-I labels are used. Although both carbon isotopes such as C14 and H3 have been used nowadays. Usually, for the pure antigen, by iodination, high specific activity for radio-labeled (125-I) antigen is prepared on its tyrosine residue(s) using method such as chloramine-T or peroxidase methods and then the radio-labeled antigen from the free-isotope using gel-filtration or HPLC. The pure antigen is used as the standard or calibrator along with the specific antibody against the antigen.

Uses of Radioimmunoassay

1. The test can be used to determine very small quantities (e.g. nanogram) of antigens and antibodies in the serum.
2. The test is used for quantitation of hormones, drugs, HBsAg, and other viral antigens.
3. Analyze nanomolar and picomolar concentrations of hormones in biological fluids.

The limitations of the RIA include:

1. The cost of equipment and reagents
2. Short shelf-life of radiolabeled compounds
3. The problems associated with the disposal of radioactive waste.

What all Radioimmunoassay Method (RIA) can be used for?

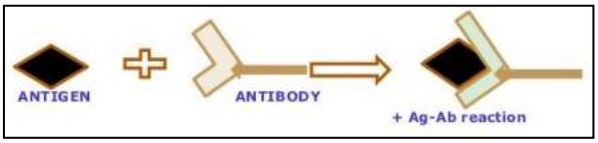
- RIA method can be used for the detection of drugs.
- Analysis of hormones, vitamins as well as other metabolite markers
- RIA can also be used to diagnose any allergy
- Detection and diagnosis of cancer
- Tracking and screening of hepatitis and leukemia viruses in the blood bank

https://www.youtube.com/watch?v=Cs1LvCyEGKg

Introduction to Antigen-Antibody Reactions

Antigen-antibody reaction is the basis of humoral immunity or antibody mediated immune response.

The interactions between antigens and antibodies are known as ***antigen–antibody reactions***. The reactions are highly specific, and an antigen reacts only with antibodies produced by itself or with closely related antigens. Antibodies recognize molecular shapes (epitopes) on antigens. Generally, the better the fit of the epitope (in terms of geometry and chemical character) to the antibody combining site, the more favourable the interactions that will be formed between the antibody and antigen and the higher the affinity of the antibody for antigen. The affinity of the antibody for the antigen is one of the most important factors in determining antibody efficacy *in vivo*.



The antigen- antibody interaction is bimolecular irreversible association between antigen and antibody. The association between antigen and antibody includes various non-covalent interactions between epitope (antigenic determinant) and variable region (V_H/V_L) domain of antibody.

Chemical Bonds Responsible for the Antigen–Antibody Reaction

The interaction between the Ab-binding site and the epitope involves exclusively non-covalent bonds, in a similar manner to that in which proteins bind to their cellular receptors, or enzymes bind to their substrates. The binding is reversible and can be prevented or dissociated by high ionic strength or extreme pH. The following intermolecular forces are involved in Ag–Ab binding:

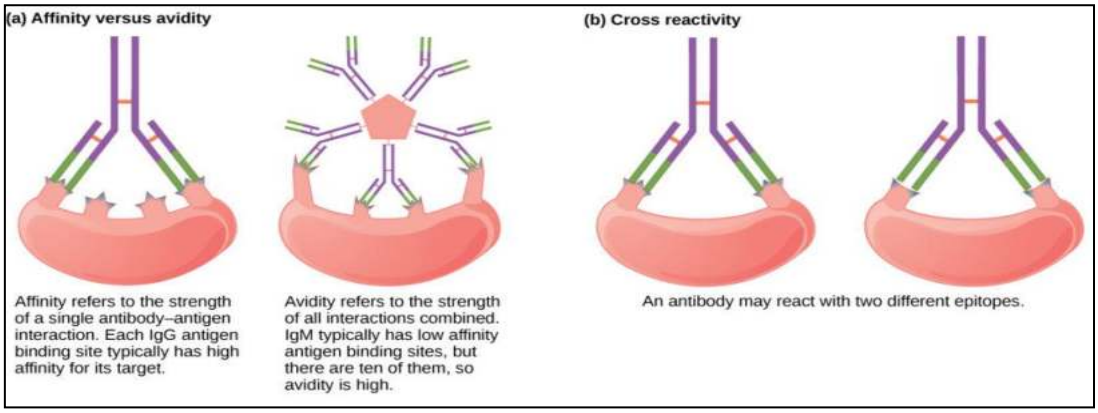
1. **Electrostatic bonds:** This result from the attraction between oppositely charged ionic groups of two protein side chains; for example, an ionized amino group (NH_4^+) on a lysine in the Ab, and an ionized carboxyl group (COO^-) on an aspartate residue in the Ag.
2. **Hydrogen bonding**: When the Ag and Ab are in very close proximity, relatively weak hydrogen bonds can be formed between hydrophilic groups (e.g., OH and C=O, NH and C=O, and NH and OH groups).
3. **Hydrophobic interactions**: Hydrophobic groups, such as the side chains of valine, leucine, and phenylalanine, tend to associate due to Vander Waals bonding and coalesce in an aqueous environment, excluding water molecules from their surroundings. As a consequence, the distance between them decreases, enhancing the energies of attraction involved. This type of interaction is estimated to contribute up to 50% of the total strength of the Ag–Ab bond.
4. **Vander Waals bonds:** These forces depend upon interactions between the “electron clouds” that surround the Ag and Ab molecules. The interaction has been compared to that which might exist between alternating dipoles in two molecules, alternating in such

a way that, at any given moment, oppositely oriented dipoles will be present in closely apposed areas of the Ag and Ab molecules.

Each of these non-covalent interactions operates over very short distance (generally about 1 Å) so, Ag-Ab interactions depends on very close fit between antigen and antibody.

Strength of Ag-Ab interactions

1. **Affinity**
   - Combined strength of total non-covalent interactions between single Ag- binding site of Ab and single epitope is affinity of Ab for that epitope.
   - Low affinity Ab: Bind Ag weakly and dissociates readily.
   - High affinity Ab: Bind Ag tightly and remain bound longer.
2. **Avidity**
   - Strength of multiple interactions between multivalent Ab and Ag is avidity. Avidity is better measure of binding capacity of antibody than affinity. High avidity can compensate low affinity.
3. **Cross reactivity**
   - Antibody elicited by one Ag can cross react with unrelated Ag if they share identical epitope or have similar chemical properties.



Types of Ag-Ab reactions – Stages

In vitro

1. Agglutination
2. Precipitation
3. Neutralization
4. Complement Fixation

In vivo

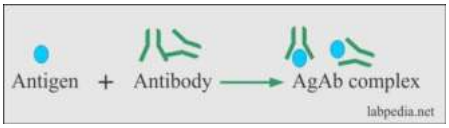
1. Agglutination
2. Precipitation
3. Complement Fixation
4. Neutralization
5. Enzyme Linked Immunosorbent Assay (ELISA)
6. RadioImmuno Assay (RIA)
7. Western Blotting
8. Fluorescent ag-ab technique

Antigen and Antibody react with each other in three stages: 3 stages

Primary stage	**Secondary stage**	**Tertiary stage**
Combination of antigen + antibody	1. Agglutination	1. Opsonization
	2. Precipitation	2. Lysis
	3. Complement activation	
	4. Neutralization	
	5. Blocking of antigen sites	

1. Primary Stage

In the primary stage, there is a combination of antigen and antibody that gives rise to antigen and antibody complex formation (Ag + Ab).



Primary stage is the initial interaction between antigen and antibody. It is rapid and **reversible,** but without any visible effects. The ionic bonds, hydrogen bonds, van der Waals forces, and hydrophobic interactions are the weaker intermolecular forces that bind antigen and antibodies together in this primary stage.

Covalent binding, which is a stronger intermolecular force between antigen and antibody, however, does not occur in this stage.

2. Secondary stage

Secondary stage is an **irreversible interaction** between anti-gen and antibody, with **visible effects,** such as agglutination, precipitation, neutralization, complement fixation, and immobilization of motile organisms. The binding between antigen and antibody during this stage occurs by covalent binding.

A single antibody is capable of causing different types of antigen–antibody reactions, and a single antigen is capable of inducing production of different classes of immunoglobulins, which differ in their biological properties.

The results of agglutination, precipitation, neutralization, and other tests are usually expressed as a titer. ***Titer*** is defined as the highest dilution of serum that gives a positive reaction in test. Higher titer means greater level of antibodies in serum. For example, a serum with a titer of 1/128 contains more antibodies than a serum with a titer of 1/8.

3. Tertiary stage

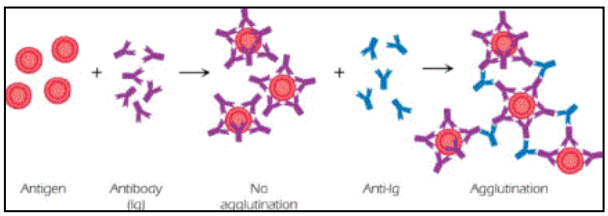
Some antigen-antibody reactions occurring in vivo initiate chain reactions that lead to neutralization or destruction / opsonizaton / lysis of injurious antigens, or to tissue damage. These are the tertiary reactions and include humoral immunity against infectious disease as well as clinical allergy and other immunological diseases.

1. Agglutination

- ✓ Agglutination is an antigen –antibody reaction where the antibody of serum causes the cellular antigen to adhere to one another to form clumps.
- ✓ It is the clumping of a particular antigen and its antibody.
- ✓ The antibody that cause agglutination are called agglutinins and particulate antigens aggregated are called agglutinogens.
- ✓ The particulate antigen includes bacterial, viruses, RBC, platelets lymphocytes, etc.
- ✓ When red blood called are agglutinated, the reaction is called Heamagglutination.
- ✓ When bacterial cells are agglutinated, the agglutination is called Bacterial Agglutination.

Simple, inexpensive, but sensitive!
Several types exist:

a) Hemagglutination of RBC's
b) Bacterial Agglutination
c) Passive Agglutination
d) Agglutination Inhibition



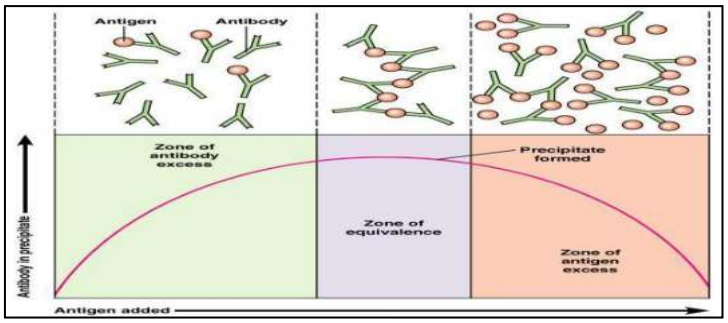
Application of Agglutination reaction:

1. Cross-matching and grouping of blood.
2. Identification of Bacteria. Ex: Serotyping of *Vibrio cholera*, Serotyping of *Salmonella Typhi* and Paratyphi.
3. Serological diagnosis of various diseases. Ex: Rapid plasma regains (RPR) test for Syphilis, Antistreptolysin O (ASO) test for rheumatic fever.
4. Detection of unknown antigen in various clinical specimens. E.g. detection of antigen of *Salmonella typhi* in the urine.

2. Precipitation

It is a type of antigen-antibody reaction, in which the antigen occurs in a soluble form. When a soluble antigen reacts with its specific antibody, at an optimum temperature and pH in the presence of electrolyte antigen-antibody complex forms insoluble precipitate. This reaction is called a precipitation reaction. A lattice is formed between the antigens and antibodies; in certain cases, it is visible as an insoluble precipitate. Antibodies that aggregate soluble antigens are called precipitins.

Antigen (Soluble)	+	Antibody -------- Precipitation (Soluble)
Antigen (Insoluble)	+	Antibody -------- Flocculation / Agglutination (Soluble)



Precipitation curve

Steps 1:

- ✓ Same proportion of Ag-Ab interact with each other by weak intramolecular forces
- ✓ Not visible
- ✓ No precipitate / lattice

Steps 2:

- ✓ Ag-Ab complement with each other and trying to cross link
- ✓ Not visible

Steps 3:

- ✓ Cross linking takes place
- ✓ Lattice formed
- ✓ Visible

Precipitation Reactions in Gels Yield Visible Precipitin Lines: Immune precipitates can form not only in solution but also in an agar matrix. When antigen and antibody diffuse toward one another in agar, or when antibody is incorporated into the agar and antigen diffuses into the antibody-containing matrix, a visible line of precipitation will form.

As in a precipitation reaction in fluid, visible precipitation occurs in the region of equivalence, whereas no visible precipitate forms in regions of antibody or antigen excess. Two types of immunodiffusion reactions can be used to determine relative concentrations of antibodies or antigens, to compare antigens, or to determine the relative purity of an antigen preparation.

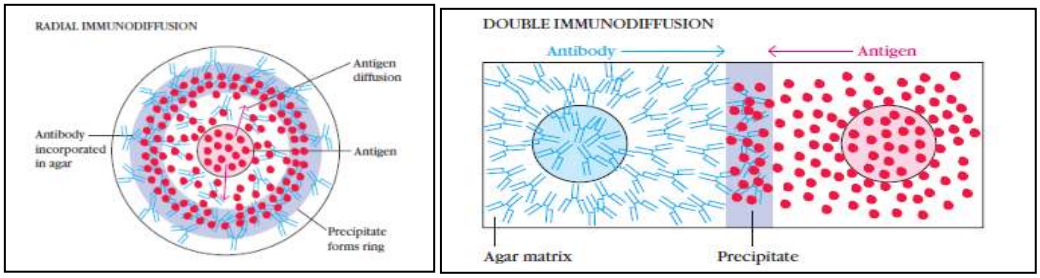
They are:

1. Radial immunodiffusion (the Mancini method)
2. Double immunodiffusion (the Ouchterlony method)

} both are carried out in a semisolid medium such as agar.

1. Radial immunodiffusion (the Mancini method)

In radial immunodiffusion, an antigen sample is placed in a well and allowed to diffuse into agar containing a suitable dilution of an antiserum. As the antigen diffuses into the agar, the region of equivalence is established and a ring of precipitation, a precipitin ring, forms around the well (Figure 6-5, upper panel). The area of the precipitin ring is proportional to the concentration of antigen. By comparing the area of the precipitin ring with a standard curve (obtained by measuring the precipitin areas of known concentrations of the antigen), the concentration of the antigen sample can be determined.



Radial immunodiffusion **Double immunodiffusion**

2. Double immunodiffusion (the Ouchterlony method)

In the Ouchterlony method, both antigen and antibody diffuse radially from wells toward each other, thereby establishing a concentration gradient. As equivalence is reached, a visible line of precipitation, a precipitin line, forms

Diagrammatic representation of radial immunodiffusion (Mancini method) & double immunodiffusion (Ochterlony method) in a gel. In both cases, large insoluble complexes form in the agar in the zone of equivalence, visible as line of precipitation (purple regions). Only the antigen (red) diffuses in radial immunodiffusion, where as both the antibody (blue) and antigen (red) diffuse in double immunodiffusion

Advantages

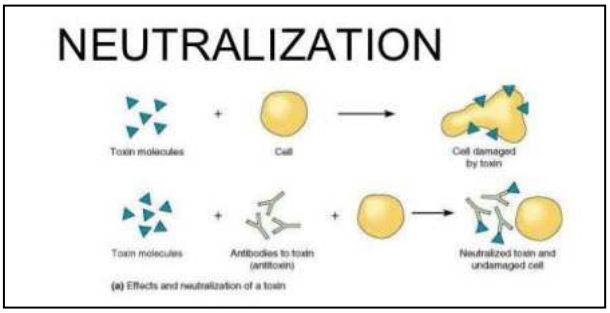
- Fairly sensitive
- High specificity

Disadvantages

- Time consuming Time consuming
- Some costly instruments are required
- High technical skill required

3. Neutralization

Bacterial exotoxins are capable of producing neutralizing antibodies (antitoxins) which play protective role in diseases such as diphtheria and tetanus.
Toxin – antitoxin neutralization can be measured in vivo and in vitro.



Neutralization is an antigen–antibody reaction in which the biological effects of viruses and toxins are neutralized by homologous antibodies known as neutralizing antibodies.

These tests are broadly of two types:

(a) Virus neutralizationν tests

(b) Toxin neutralization tests

Neutralizing antibodies block or distort the antigenν sufficiently, so that pathogen fails to exert its biological activity. Neutralization reactions can occur in vitro or in vivo.ν Laboratory animals or tissue culture cells are used asν "indicator systems" in neutralization tests. The toxin or virus to be assayed should have known effects on the indicator system which is neutralized by antibodies.

Toxin neutralization tests

Toxin neutralization tests are based on the principle that biological action of toxin is neutralized on reacting with specific neutralizing antibodies called antitoxins.

Examples of toxin neutralization tests include:

In vivo:
(a) Schick test to demonstrate immunity againstν diphtheria and

(b) Clostridium welchii toxin neutralization test in guineaν pig or mice.

In vitro:

(a) Antistreptolysin O test

(b) Nagler reaction used for rapid detection of *Clostridium welchii*

Virus Neutralization Test

Neutralization of viruses by their specific antibodies are calledv virus neutralization tests. Inoculation of viruses in cell cultures, eggs, and animalsv results in the replication and growth of viruses. When virus specific neutralizing antibodies are injected into these systems, replication and growth of viruses is inhibited. This forms the basis of virus neutralization test. Neutralizing antibodies may interfere with virion binding tov receptors, block uptake into cells, prevent uncoating of the genomes in endosomes, or cause aggregation of virus particles. Neutralizing antibody is directed against the surface proteinsv of the virus. Antibodies formed against internal components of the virus do not neutralize the infectivity of the virus; rather such non-neutralizing antibodies may enhance their infectivity

4. Complement Fixation Test- Steps, Advantages and Disadvantages

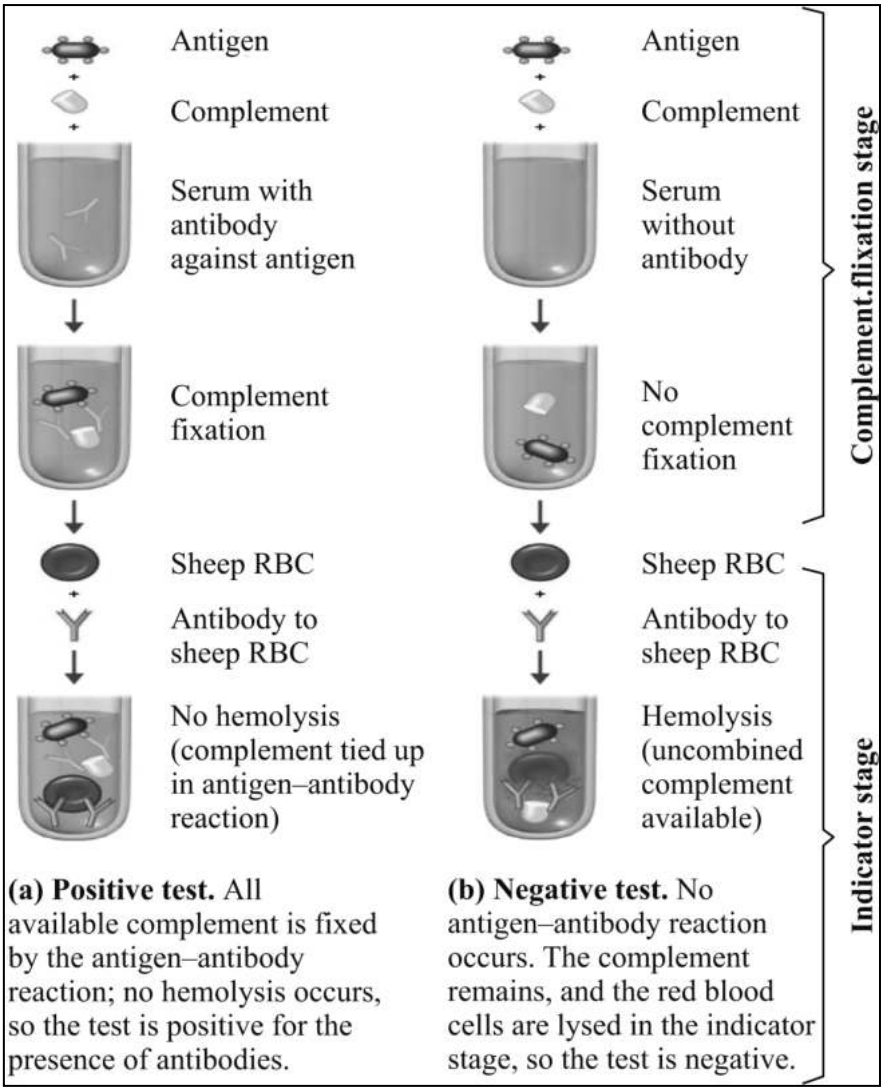
It is a classic method for demonstrating the presence of antibody in patient serum. It is based on the principle that antigen-antibody complex fixes the complement. As coupling of complement has no visible effects or changes, it is necessary to use an indicator system consisting of sheep RBC and coated with anti-sheep RBC antibody. Complement lyses antibody coated RBC.

Steps of Complement Fixation Test

Step 1: A known antigen and inactivated patient's serum are incubated with a standardized, limited amount of complement. If the serum contains specific, complement activating antibody the complement will be activated or fixed by the antigen-antibody complex. However, if there is no antibody in the patient's serum, there will be no formation of antigen-antibody complex, and therefore complement will not be fixed. But will remain free.

Step 2: The second step detects whether complement has been utilized in the first step or not. This is done by adding the indicator system. If the complement is fixed in the first step owing to the presence of antibody there will be no complement left to fix to the indicator system. However, if there is antibody in the patient's serum, there will be no antigen-antibody complex, and therefore, complement will be present free or unfixed in the mixture. This unfixed complement will now react with the antibody- coated sheep red blood cells to bring about their lysis.

Thus, no lysis of sheep red blood cells (positive CFT) indicates the presence of antibody in the test serum, while lysis of sheep red blood cells (Negative CFT) indicates the absence of antibody in the serum.



Advantages of Complement Fixation Test

1. Ability to screen against a large number of viral and bacterial infections at the same time.
2. Economical.

Disadvantages of Complement Fixation Test

1. Not sensitive – cannot be used for immunity screening.
2. Time-consuming.
3. Often non-specific e.g. cross-reactivity between Herpes Simplex Virus and Voricella Zoster Virus.

https://www.youtube.com/watch?v=EfLJGR0U2gQ

5. ELISA- Principle, Types and Applications

Enzyme-linked Immunosorbent Assay (shortened as ELISA) is used to identify peptides, proteins, antibodies and hormones. Also, called as enzyme immunoassay (EIA), ELISA finds use in the fields of biotechnology and medicine as a diagnostic tool. Mainly, antibodies and

colour changes are used to identify target substances. Also, ELISAs are useful in measuring antigen and antibody concentration.

Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) are both widely used as diagnostic tools in medicine and as quality control measures in various industries; they are also used as analytical tools in biomedical research for the detection and quantification of specific antigens or antibodies in a given sample

How does ELISA work?

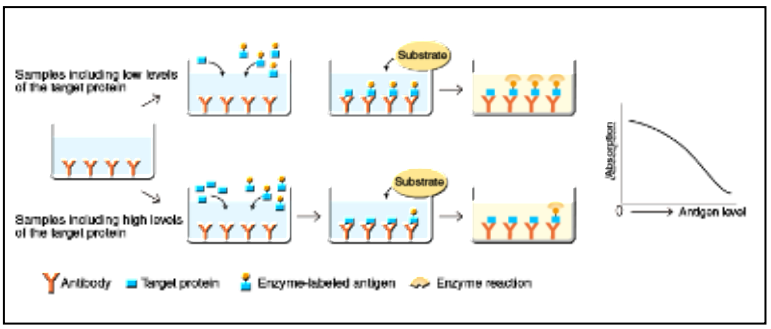
ELISA is based on specific antigen-antibody reaction and usually involves immobilizing antibodies or antigens to a 96-well or 384-well plate. The basic steps of ELISA:

ELISAs are typically performed in 96-well polystyrene plates. The serum is incubated in a well, and each well contains a different serum.

1. Immobilization of the target proteins/antigens on the surface of a microplate
2. Washing unbound/excess proteins/antigens from the plate
3. Adding a labelled antibody which will subsequently bind the target antigen/protein present in the plate
4. Washing unbound (excess) antibodies off the plate
5. Adding enzyme-specific substrates that will react with the enzyme and produce a colored product, which can be measured colorimetrically using a microplate reader.

Common enzymes used in ELISA: Horse radish peroxidase (HRP) or alkaline phosphatase
Common substrates include: Tetramethylbenzidine (TMB) and 2, 2′-azino-bis-3-Ethylbenzthiazoline-6-sulphonic acid (*ABTS*)

Duplicate or triplicate sampling is generally preferred and different concentrations of the sample are used to ensure biologically acceptable range of detection.

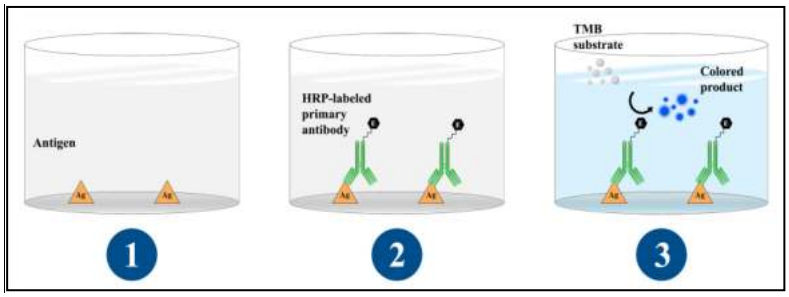


ELISA – Types:

1. Direct ELISA
2. Indirect ELISA
3. Sandwich ELISA
4. Competitive ELISA

1. **Direct ELISA-** *Antigen screening; detect soluble antigen*

The detection via direct ELISA procedure is much faster as compared to other ELISA techniques due to limited steps followed. This technique allows for coating of target protein / antigen directly to wells of microtitre plate which is followed by the addition of enzyme labelled primary antibody that detects the antigen that is complementary. The assay is beneficial as it is minimally error prone due to the implementation of a lesser number of steps requiring only a handful of reagents. There *is no requisite of cross-reacting secondary antibody in this technique.* But this technique poses a few disadvantages with regards to its specificity.



Advantages

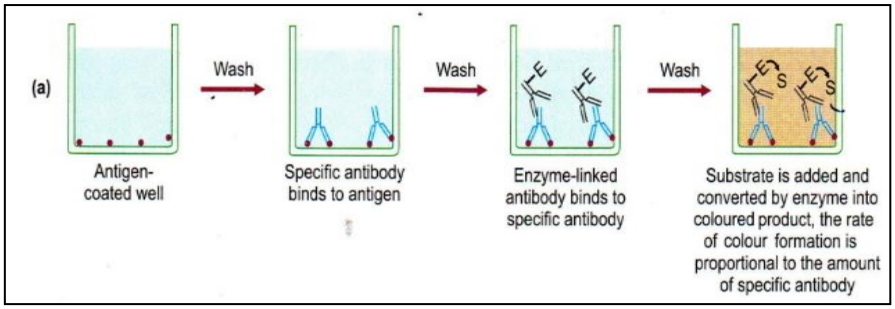
- ✓ Fast and minimal steps involved in the procedure.
- ✓ Minimum precursor requirement makes it less error prone.

Disadvantages

- ✓ The immobilization of the antigen is not specific due to which background related issues are seen.
- ✓ Offers less flexibility in terms of primary antibody.
- ✓ The absence of signal amplification reduces sensitivity.

2. Indirect ELISA- *Antibody screening; epitope mapping*

Antibody can be detected or quantitatively determined by indirect ELISA. In this technique, antigen is coated on the microtiter well. Serum or some other sample containing primary antibody is added to the microtiter well and allowed to react with the coated antigen. Any free primary antibody is washed away and the bound antibody to the antigen is detected by adding an enzyme conjugated secondary antibody that binds to the primary antibody. Unbound secondary antibody is then washed away and a specific substrate for the enzyme is added. Enzyme hydrolyzes the substrate to form colored products. The amount of colored end product is measured by spectrophotometric plate readers that can measure the absorbance of all the wells of 96-well plate.



Advantages

- ✓ Offers high sensitivity and flexibility as the number of secondary antibodies can bind to a primary antibody and one type of secondary antibody can label different primary antibodies
- ✓ It is cheaper as there is a requirement of fewer labeled antibodies.

Disadvantages

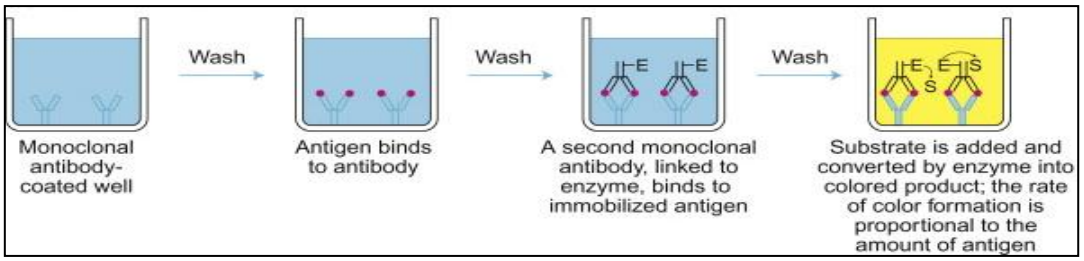
- ✓ Higher signal to noise ratio.
- ✓ Time consuming and extra labour required.

3. Sandwich ELISA

An antibody to a target protein is immobilized on the surface of microplate wells and incubated first with the target protein and then with another target protein-specific antibody, which is labeled with an enzyme. After washing, the activity of the microplate well-bound enzyme is measured. The immobilized antibody (orange) and the enzyme-labeled antibody (green) must recognize different epitopes of the target protein.

(OR)

In this system, the target antigen is detected via anchoring between two antibodies, which recognize different epitopes, or the so-called sandwich system. Sandwich ELISA starts from the immobilization of an antibody, called a capture antibody, on the microtiter plate. After blocking the plate surface to avoid non-specific adsorption of other proteins, the antigen in the sample is allowed to react with the immobilized capture antibody, and the antigen bound to the capture antibody is then sandwiched with an enzyme-labeled antibody for colour development. This direct system can be modified to the indirect system by using primary and enzyme-labeled secondary antibodies. The signal increases with increasing amount of antigen. As two antibodies containing different epitopes are required against the target antigen, sandwich ELISA is generally suitable for measuring macromolecules with some exceptions.



Advantages

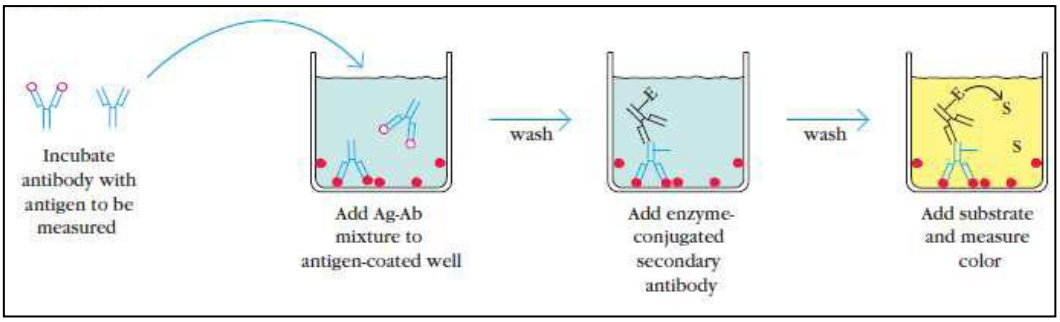
- ✓ Offers high sensitivity compared to direct or indirect ELISA
- ✓ Introduces highly specific reaction due to the involvement of two antibodies for antigen detection.
- ✓ Both direct and indirect technique is implemented in detection.

Disadvantages

- ✓ Optimization in terms of antibody becomes problematic due to cross-reactivity issues.
- ✓ For recognition of a specific epitope, only monoclonal antibodies can be applied as matched pairs.
- ✓ To procure monoclonal antibodies it is a tedious process in case of matched pairs and are more expensive than polyclonal antibodies.

5. Competition/Inhibition ELISA

In this procedure, a reference antigen is immobilized on the plate surface and biological sample pre-incubated with a specific amount of labeled antibody is added to the plate. The amount of antigen present in the sample will determine the amount of unbound or free antibodies available for binding the reference antigen in the plate.



Advantages

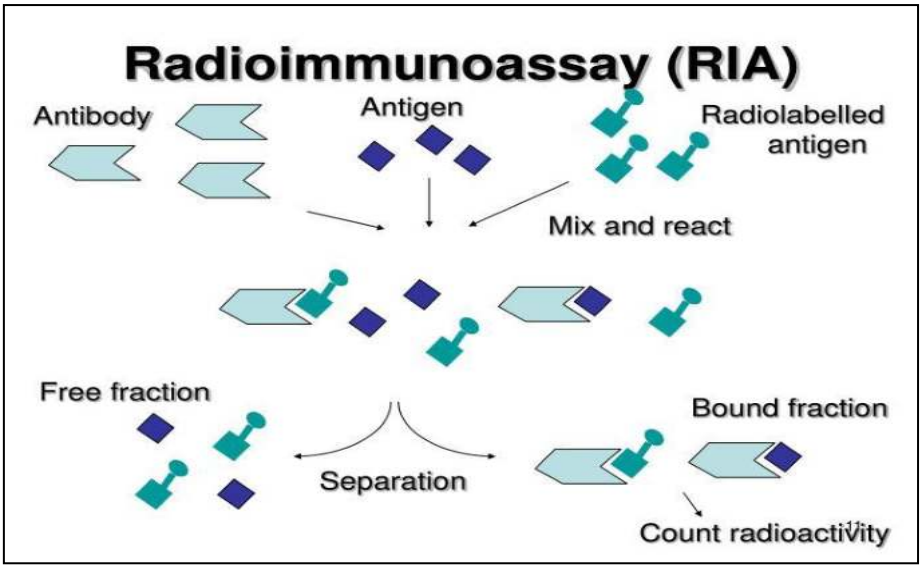
- ✓ Negligible sample processing is required and can be applicable to crude samples
- ✓ Less sensitive to experimental errors.
- ✓ Good reproducibility and flexibility.
- ✓ The main advantage of competition ELISA is its high sensitivity to compositional differences in complex antigen mixtures, even when the specific detecting antibody is present in relatively small amounts

Disadvantages

- ✓ Basic ELISA limitations apply

6. Radioimmunoassay

Radioimmunoassay (RIA) is is an *in vitro* assay and based on the principle of all immunoassays which is the recognition of an antigen present in a sample by antibodies directed against this antigen. The principle of radioimmunoassays is very similar to that of competitive ELISA and allows quantification of small molecules, peptides and proteins in biological samples.



Requirements of RIA Radioimmunoassay involves three components:

Pure antigen, radiolabeled antigen, and antiserum (antibody). In addition a separation technique is essential to estimate the distribution of radioactivity in the free and bound fractions. The sensitivity of an assay depends to a large extent on the quality of these components and choice of a suitable separation technique.

Radioactivity:

Radioisotopes are one of the main factors for immunodiagnostic technology. Usually, Iodine isotope 125-I labels are used. Although both carbon isotopes such as C14 and H3 have been used nowadays. Usually, for the pure antigen, by iodination, high specific activity for radio-labeled (125-I) antigen is prepared on its tyrosine residue(s) using method such as chloramine-T or peroxidase methods and then the radio-labeled antigen from the free-isotope using gel-filtration or HPLC. The pure antigen is used as the standard or calibrator along with the specific antibody against the antigen.

Uses of Radioimmunoassay

1. The test can be used to determine very small quantities (e.g. nanogram) of antigens and antibodies in the serum.
2. The test is used for quantitation of hormones, drugs, HBsAg, and other viral antigens.
3. Analyze nanomolar and picomolar concentrations of hormones in biological fluids.

The limitations of the RIA include:

1. The cost of equipment and reagents
2. Short shelf-life of radiolabeled compounds
3. The problems associated with the disposal of radioactive waste.

What all Radioimmunoassay Method (RIA) can be used for?

- RIA method can be used for the detection of drugs.
- Analysis of hormones, vitamins as well as other metabolite markers
- RIA can also be used to diagnose any allergy
- Detection and diagnosis of cancer
- Tracking and screening of hepatitis and leukemia viruses in the blood bank

https://www.youtube.com/watch?v=Cs1LvCyEGKg

Antigen-Antibody Reactions

BSc Nursing 2nd year

Dr. Mohit Bhatia
Assistant Professor
Department of Microbiology
AIIMS, Rishikesh

Learning objectives

By the end of this session students should be able to know:

- General properties of antigen antibody reactions
- Types of antigen antibody reactions
- Conventional immunoassays
- Overview of newer techniques

Antigen-antibody reaction

- Bimolecular association where the antigen and antibody combine with each other specifically and in an observable manner.
  - Similar to an enzyme-substrate interaction.
  - Only difference is, it does not lead to an irreversible alteration in either antibody or in antigen.

PROPERTIES OF ANTIGEN ANTIBODY REACTIONS

1. **Specific:**
   - Ag-Ab reaction involves specific interaction of epitope of an antigen with the corresponding paratope of its homologous antibody.
   - Exception is the cross reactions which may occur due to sharing of epitopes among different antigens.
   - In such case, antibody against one antigen can cross react with a similar epitope of a different antigen.

PROPERTIES OF ANTIGEN ANTIBODY REACTIONS

2. **Non-covalent interactions:**
   - Union of antigen and antibody requires formation of large number of non-covalent interactions between them such as:
     - Hydrogen bonds
     - Electrostatic interactions
     - Hydrophobic interactions
     - van der Waals forces

PROPERTIES OF ANTIGEN ANTIBODY REACTIONS

- 3. Strength- The strength or the firmness of the association is influenced by the affinity and avidity of the antigen-antibody interaction.

  - Affinity
    - Refers to sum total of non covalent interactions between a single epitope of an antigen with its corresponding paratope present on antibody.
    - Affinity can be measured by two methods- i) by equilibrium dialysis and ii) by surface plasmon resonance method

PROPERTIES OF ANTIGEN ANTIBODY REACTIONS

- Avidity: Term used to describe the affinities of all the binding sites when multivalent antibody reacts with a complex antigen carrying multiple epitopes.
  - When a complex antigen carrying multiple epitopes reacts with a multivalent antibody; the total strength (i.e. avidity) would be much higher than the individual affinity at each binding site.
  - Avidity is not exactly equal but lower than the sum of all affinities.
  - This difference is primarily due to geometry of antigen antibody binding.

PROPERTIES OF ANTIGEN ANTIBODY REACTIONS

- Avidity is a better indicator of strength of an antigen antibody reaction.

- Avidity of an antibody can compensate for its low affinity.
  - For example, IgM has a low affinity than IgG but it is multivalent (10 valencies), therefore has a much higher avidity. It can bind to an antigen more effectively than IgG.

Diagnostic Use of Ag-Ab reactions

- Specific and observable
- The diagnostic tests based on antigen-antibody reactions are called as immunoassays
- Immunoassays can be broadly categorized into two types-
  - Antigen detection assays
  - Antibody detection assays

Qualitative assays

- Result is read as ‘positive’ or ‘negative’ based on presence or absence of antigen or antibody in the clinical specimen.

Disadvantages

- Number of antigen or antibody molecules in the reaction are disproportionate to each other.
  - If either antigen or antibody are present in higher quantity - false negative.
    - To rule out a false negative result, it is ideal to test the series of diluted sera (quantitative test) instead of just testing the one specimen of undiluted serum.

Quantitative assays

- When the qualitative test turns positive, the exact amount of antibody in serum can be known by serial dilution of the patient's serum and mixing each dilution of the serum with a known quantity of antigen.

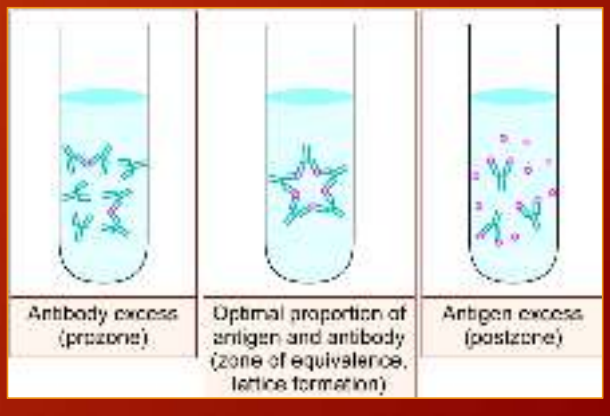
- The measurement of antibody is expressed in terms of units or more commonly titer.

Quantitative assays

- Quantitative tests are more reliable as they can differentiate between true negative and false negative results.

Marrack's or Lattice hypothesis

- Ag-Ab reaction is weak or fails to occur when the number of antigen and antibodies are not proportionate to each other.
- Prozone phenomenon - In the earlier test tubes, antibodies are excess, hence the Ag-Ab reaction does not occur.
- Post zone phenomenon - In the later test tubes, antigen is excess, hence the Ag-Ab reaction fails to occur.



Marrack's or Lattice hypothesis

- In prozone/post zone - Lattice does not enlarge, due to inhibition of lattice formation by the excess antibody or antigen respectively, as the valencies of the antibody and the antigen respectively, are fully satisfied.

Evaluation of Immunoassays

- Sensitivity and specificity are the two most important statistical parameters.
- Sensitivity is defined as ability of a test to identify correctly all those who have the disease i.e. true positives.
- Sensitivity is calculated as $= \frac{\text{True positives}}{\text{True positives + False negatives}}$

Evaluation of Immunoassays

- Specificity is defined as ability of a test to identify correctly all those who do not have disease i.e. true negatives.
- Specificity is calculated as = $\frac{\text{True negatives}}{\text{True negatives + False positives}}$

Types of antigen-antibody reactions

Conventional techniques -

- Precipitation reaction
- Agglutination reaction
- Complement fixation test
- Neutralization test

Newer techniques -

- Enzyme linked immunosorbent assay (ELISA)
- Enzyme linked fluorescent assay (ELFA)
- Immunofluorescence Assay (IFA)
- Radioimmunoassay (RIA)
- Chemiluminescence-linked immunoassay (CLIA)
- Immunohistochemistry
- Rapid tests-
  - Lateral flow assay (Immunochromatographic test)
  - Flow through assay
- Western blot
- Immunoassays using electron microscope

PRECIPITATION REACTION

- **Definition-**When a *soluble antigen* reacts with its antibody in the presence of optimal temperature, pH and electrolytes (NaCl), it leads to formation of the antigen-antibody complex in the form of:
  - Insoluble precipitate band when gel containing medium is used

    or
  - Insoluble floccules when liquid medium is used (precipitate remains suspended as floccules)

Precipitation in liquid medium

- **Ring test**: In a narrow tube (e.g. capillary tube), antigen solution is layered over an antiserum
  - Precipitate ring appears at the junction of two liquids.
  - Example: Streptococcal grouping by Lancefield technique, and Ascoli's thermoprecipitin test done for anthrax.

Precipitation in liquid medium

- **Flocculation test**- When a drop of antigen is mixed with a drop of patient's serum, then floccules appear.
  - Examples of slide flocculation test- VDRL and RPR tests used for diagnosis of syphilis
  - Examples of tube flocculation test- Kahn test used previously for syphilis

Precipitation in gel (Immunodiffusion)

- Using 1% soft agarose gel for precipitation reaction has many advantages over liquid medium-
  - Results in formation of clearly visible bands instead of floccules that can be preserved for longer time.
  - Can differentiate individual antigens from a mixture as each antigen forms a separate band after reacting with specific antibody.

Principle of Immunodiffusion

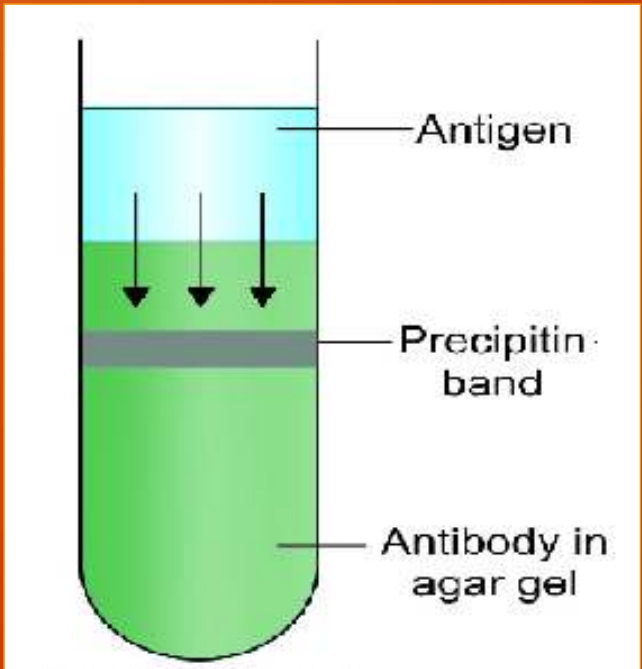
- Whether only Ag diffuses (single diffusion) or both Ag and Ab diffuse (double diffusion)

- Whether Ag or Ab diffuses in *one dimension* (i.e. vertical diffusion when test is done on a tube layered with gel) or *two dimensions* (i.e. diffusion in both X and Y axis when test is done on a slide or a petri dish layered with gel)

Types of immunodiffusions in gel

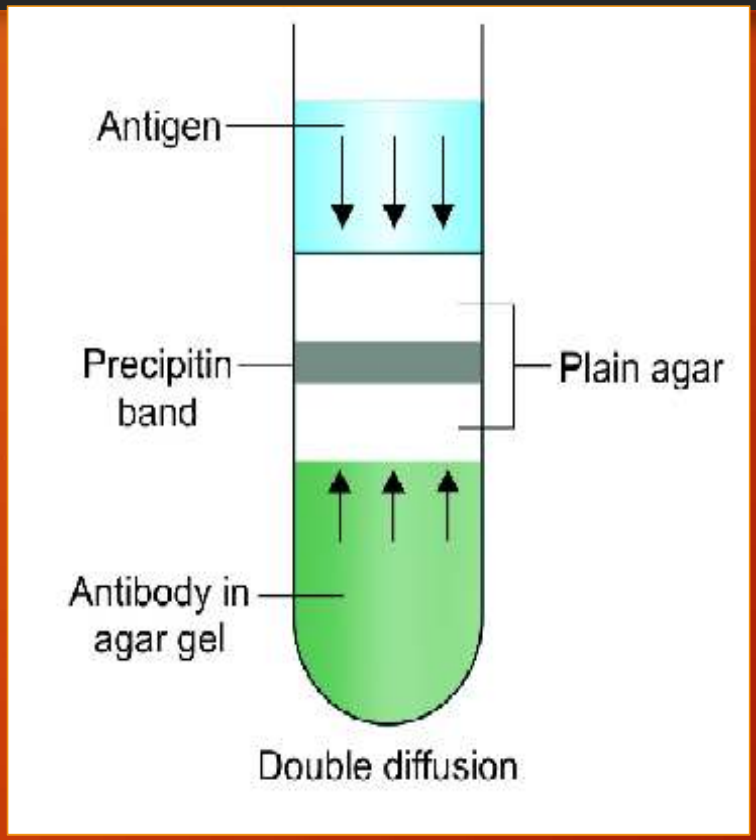
1. Single diffusion in one dimension (Oudin procedure)
2. Double diffusions in one dimension (Oakley- Fulthorpe procedure)
3. Single diffusion in two dimensions (Radial immunodiffusion)
4. Double diffusions in two dimensions (Ouchterlony procedure)

Single diffusion in one dimension (Oudin procedure)

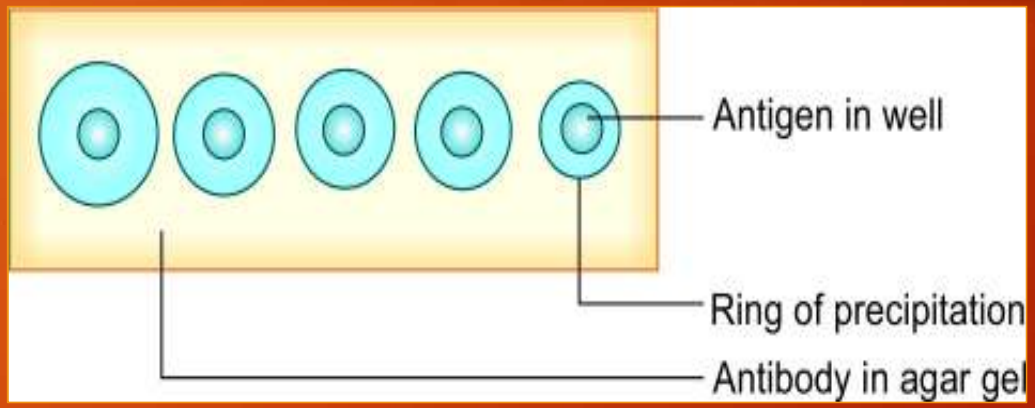


Single diffusion

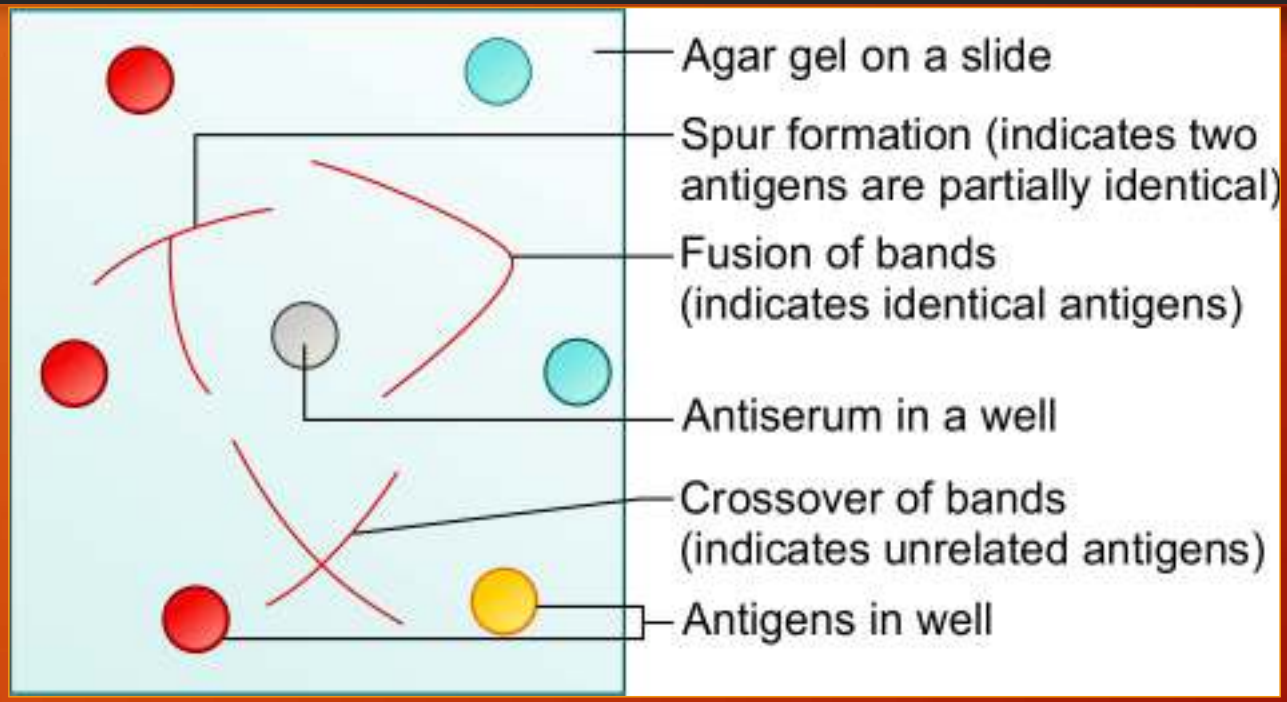
Double diffusions in one dimension (Oakley- Fulthorpe procedure)



Single diffusion in two dimensions (Radial immunodiffusion)



Double diffusions in two dimensions (Ouchterlony procedure)



Double diffusions in two dimensions (Ouchterlony procedure)

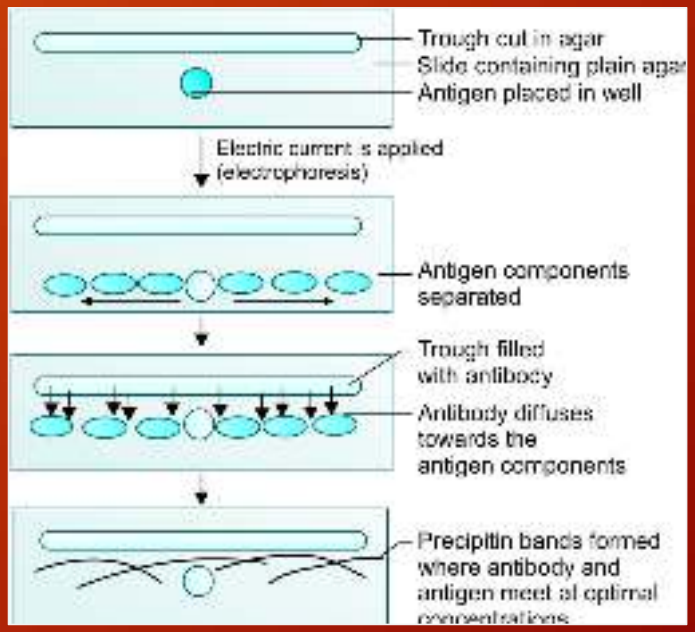
- Examples of double diffusions in two dimensions include:
  - **Elek's test** for detecting toxin of *Corynebacterium diphtheria*.
  - **Eiken test** to detect toxin of *Escherichia coli*

Precipitation in gel in presence of electric current

1. Electroimmunodiffusion (EID)
2. CIEP (Countercurrent immunoelectrophoresis)
3. Rocket electrophoresis

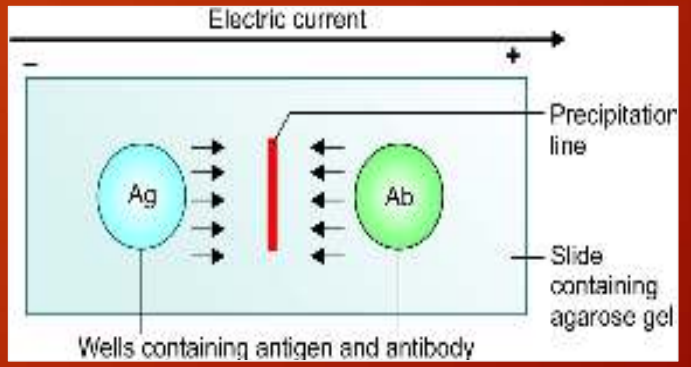
Electroimmunodiffusion

- Electric current is applied to a slide layered with gel.
- Helps in identification and approximate quantitation of various proteins present in the serum.



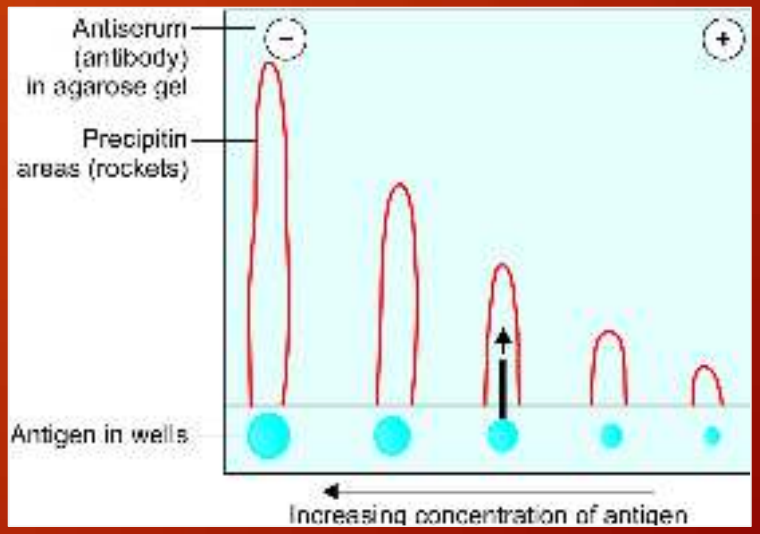
Counter-current immunoelectrophoresis

- Test is even faster (takes 30 minutes) and more sensitive than EID.
- Popular in the past - detecting alpha fetoprotein in serum and capsular antigens of *Cryptococcus* and meningococcus in the cerebrospinal fluid.



Rocket electrophoresis

- One-dimensional single electroimmunodiffusion test.
- Was mainly used in the past for quantitative estimation of antigens.



AGGLUTINATION REACTION

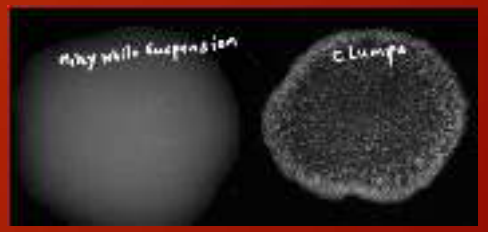
- **Definition-**When a **particulate** or **insoluble** antigen is mixed with its antibody in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated.
- **Advantage**:
  - More sensitive than precipitation test.
  - Clumps are better visualized and interpreted than bands or floccules.

AGGLUTINATION REACTION

- Wide diagnostic applications of agglutination reactions.
- Classified as:
  - Direct agglutination reactions.
  - Indirect (passive) agglutination reactions.
  - Reverse passive agglutination reactions.
- Performed either on a slide, or in tube or in card or some time in microtiter plates.

Direct agglutination test

- Antigen directly agglutinates with the antibody.
- ***Slide agglutination***:
  - Performed to confirm the identification and serotyping of bacterial colonies grown in culture.
  - Method used for blood grouping and cross matching.



Tube agglutination

- Standard quantitative test for estimating antibody in serum.
- ***Antibody titre*** can be estimated as the highest dilution of the serum which produces a visible agglutination.

Tube agglutination- Applications

- Enteric fever (Widal test)
- Acute brucellosis (Standard agglutination test)
- Coombs Antiglobulin test (see below)
- Heterophile agglutination tests:
  - Typhus fever (Weil Felix reaction)
  - Infectious mononucleosis (Paul Bunnell test)
  - *Mycoplasma pneumoniae* (Cold agglutination test)

Microscopic agglutination test (MAT)

- Agglutination test is performed on a microtiter plate.
- Result is read under a microscope.
- MAT is done for leptospirosis.

Indirect or passive agglutination test

- Indirect hemagglutination test (IHA)
- Latex agglutination test (LAT)

Indirect hemagglutination test (IHA)

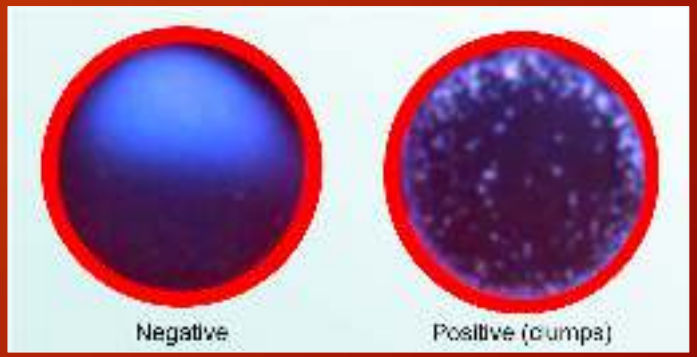
- Passive agglutination test where RBCs are used as carrier molecules.
- IHA was used widely in the past, but is less popular at present.

Latex agglutination test (LAT) for antibody detection

- Polystyrene latex particles (0.8 – 1 µm in diameter) are used as carrier molecules which are capable of adsorbing several types of antigens.
- For better interpretation of result, the test is performed on a black colour card.
- Drop of patient's serum (containing antibody) is added to a drop of latex solution coated with the antigen and the card is rocked.

Latex agglutination test (LAT) for antibody detection

- Positive result is indicated by formation of visible clumps.
- LAT is one of the most widely used tests at present as it is very simple and rapid.
- Used for detection of ASO (antistreptolysin O antibody).



Reverse passive agglutination test (for antigen detection)

- Antibody is coated on a carrier molecule which detects antigen in the patient's serum.

Test	Carrier molecule	Clinical applications
RPHA (Reverse passive hemagglutination assay)	RBCs	• Detection of hepatitis B surface antigen (HBsAg) – not used now.
Latex agglutination test	Latex particles	• Detection of CRP (C reactive protein), RA (rheumatoid arthritis factor), capsular antigen detection in CSF (for pneumococcus, meningococcus and *Cryptococcus*) and streptococcal grouping.

Reverse passive agglutination test (for antigen detection)

Test	Carrier molecule	Clinical applications
Coagglutination test	*Staphylococcus aureus*	• Used in past for antigen detection (e.g. *Salmonella* antigen detection from blood and urine). • Obsolete at present.

Hemagglutination test

- Refers to the agglutination tests that use red blood cells (RBCs) as source of antigen.
- Various types of hemagglutination tests include:
  - ***Direct hemagglutination test***
  - ***Indirect hemagglutination test***

Direct hemagglutination test

- Serum antibodies directly agglutinate with surface antigens of RBCs to produce a matt. Examples include-
  - Paul Bunnell test
  - Cold agglutination test
  - Blood grouping
  - Coombs test or Antiglobulin test

Coombs test or **Antiglobulin test**

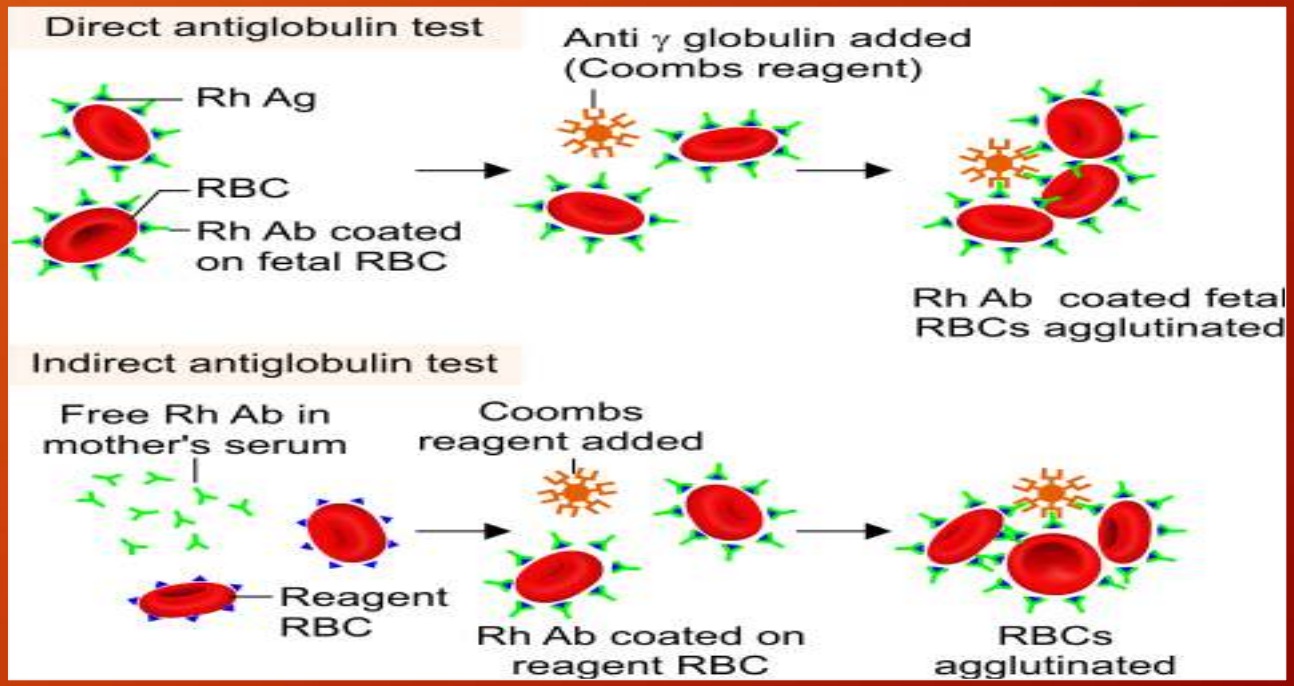
- Performed to diagnose Rh incompatibility by detecting Rh antibody from mother's and baby's serum.
- *Rh incompatibility* is a condition when an Rh negative mother (Rh Ag -ve and Rh Ab -ve) delivers a Rh positive baby (Rh Ag +ve and Rh Ab -ve).
- During birth, some Rh Ag +ve RBCs may pass from fetus to the maternal circulation and may induce Rh Ab formation in mother and affect future Rh positive pregnancies.

Coombs test or Antiglobulin test

- Rh antibodies are ***incomplete*** or ***blocking*** antibodies of IgG type.
- They can cross placenta and bind to Rh Ag on fetal RBCs.
- Does not result in agglutination; instead they block the sites on fetal RBCs.
- Such reaction can be visualized by adding ***Coombs reagent*** *(antiglobulin* or *antibody to human IgG*) which can bind to Fc portion of Rh Ab bound on RBCs, resulting in visible agglutination.

Coombs test or Antiglobulin test - Types

- *Direct Coombs test*
- *Indirect Coombs test*



Viral hemagglutination test

- In strict sense, it is not an antigen antibody reaction. The hemagglutinin antigens (HA) present on surface of some viruses
- Hemagglutinating viruses (e.g. influenza virus) can agglutinate with the receptors present on the surface of RBCs.

Technical issues in agglutination reactions

- Two main problems pertaining to agglutination - can cause **false negative** agglutination test.
  - *Prozone phenomenon*
  - *Blocking antibodies*

COMPLEMENT FIXATION TEST

- Detects the antibodies in patient's serum that are capable of fixing with complements. It was once very popular, now is almost obsolete.
- **Applications:**
  - **Wasserman test-**Syphilis
  - Was also widely used for detection of complement fixing antibodies in *Rickettsia, Chlamydia, Brucella, Mycoplasma infections and some viral* infections, such as arboviruses, rabies, etc.
  - Complements are also used for various serological tests other than CFT, such as:
    - *Treponema pallidum immobilization test*
    - Sabin-feldman dye test for *Toxoplasma*
    - Vibriocidal antibody test.

NEWER TECHNIQUES

- Uses a detector molecule to label antibody or antigen which in turn detects the corresponding antigen or the antibody in the sample by producing a visible effect.

- Most of the newer techniques use the same principle, but they differ from each other by the type of labelled molecule used and the type of visible effect produced.

Immunoassays and the types of molecule used for labelling

Abbreviation	Immunoassay method	Molecules used for labelling	Type of visible effect
ELISA	Enzyme linked immunosorbent assay	Enzyme- substrate-chromogen complex	Colour change is detected by spectrophotometer
ELFA	Enzyme linked fluorescent assay	Enzyme- substrate	Fluorometric detection
IFA	Immunofluorescence Assay	Fluorescent dye	Emits light, detected by fluorescence microscope
RIA	Radioimmunoassay	Radioactive isotope	Emits β and γ radiations, detected by β and γ counters
CLIA	Chemiluminescence-linked immunoassay	Chemiluminescent compounds	Emits light, detected by luminometer

Immunoassays and the types of molecule used for labelling

Abbreviation	Immunoassay method	Molecules used for labeling	Type of visible effect
IHC	Immunohistochemistry	Enzyme or Fluorescent dye	Color change (naked eye) or Fluorescence microscope
WB	Western blot	Enzyme	Colour band (naked eye)
Rapid test	Immunochromatographic test	Colloidal gold or silver	Colour band, (naked eye)
	Flow through assay	Protein A conjugate	Colour band, (naked eye)
IEM	Immunoferritin electron microscopy	Electron dense molecules (e.g ferritin)	Appears as black dot under electron microscope

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

- Enzyme immunoassay (EIA) is a term used to describe all the tests that detect either antigen or antibodies or haptens in the specimen, by using enzyme-substrate system for detection.

- They can be classified as below:
  - **Homogenous EIA**
  - **Heterogeneous EIA**

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

- **Homogenous EIA**
  - All reagents are added at one step.
  - Used for detection of haptens such as drugs (e.g. opiates, cocaine), but not for detection of microbial antigens and antibodies

- **Heterogeneous EIA**
  - **Involves multiple steps with** different reagents being added at every step.
  - Used for detection of antigens and antibodies.
  - ELISA is a classical example.

ELISA (ENZYME LINKED IMMUNOSORBENT ASSAY)

- So named because of two of its components-
  - Immunosorbent- Adsorbing material used (e.g. polystyrene, polyvinyl) that specifically adsorbs the antigen or antibody present in serum.
  - Enzyme is used to label one of the components of immunoassay (i.e. antigen or antibody).

Substrate-chromogen system

- Substrate- chromogen system is added at the final step of ELISA.
- Enzyme-substrate reaction in turn activates the chromogen to produce a colour.
- Sometimes, the substrate is chromogenic in nature (e.g. pNPP), on reaction with the enzyme, it changes its colour.

Substrate-chromogen system

- Colour change - detected by spectrophotometry in an ELISA reader.
- Intensity of the colour is directly proportional to the amount of detection molecule (Ag or Ab) present in test serum.

Enzyme	Substrate	Chromogen
Horseradish Peroxidase	Hydrogen peroxide	Tetramethyl benzidine (TMB)
Urease	Urea	Bromocresol
ß-Galactosidase	ONPG	ONPG
Alkaline Phosphatase	pNPP*	pNPP*

ELISA

- (Ag-Ab complex)-enzyme + substrate → activates the chromogen → colour change → detected by spectrophotometry

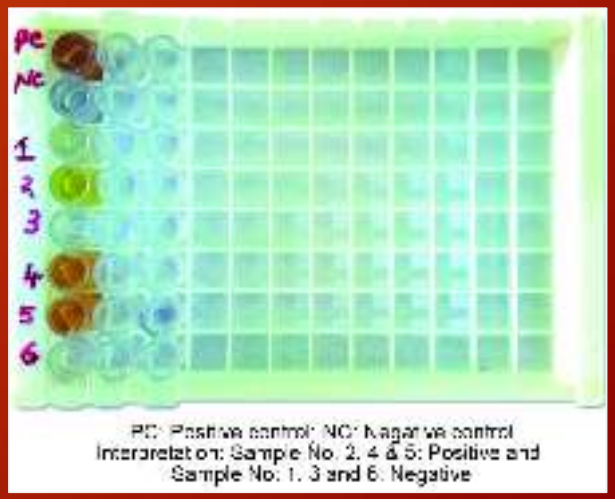
ELISA reader (Biorad)

ELISA washer

Automated ELISA
(EVOLIS system, Biorad)

ELISA

- Performed on a microtiter plate containing 96 wells (micro-ELISA) or less commonly performed in tubes (macro-ELISA).
- The microtiter plate or the tubes are made up of polystyrene, polyvinyl or polycarbonate material.



PC: Positive control; NC: Negative control
Interpretation: Sample No. 2, 4 & 5: Positive and
Sample No: 1, 3 and 6: Negative

ELISA

- ELISA kits are commercially available; contain all the necessary reagents (such as enzyme conjugate, dilution buffer, substrate/chromogen etc).

- Procedure involves a series of steps done sequentially; at each step, a reagent is being added, and then incubated followed by washing of the wells (manually or by automated ELISA washer).

Types of ELISA

ELISA type	Used for detection of	Enzyme is labeled with
Direct ELISA	Antigen	Primary antibody
Indirect ELISA	Antibody or antigen	Secondary antibody
Sandwich ELISA	Antigen	Primary antibody in sandwich direct ELISA Secondary antibody in sandwich indirect ELISA
Competitive ELISA	Antigen or antibody	Secondary antibody
ELISPOT	Cells producing antibody or cytokine	Primary antibody

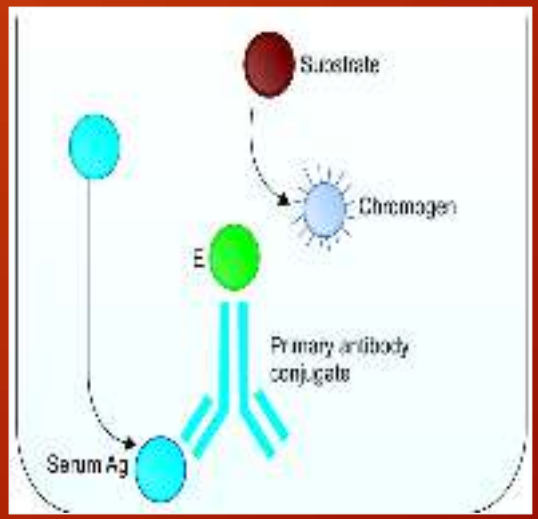
Direct ELISA

- Used for detection of antigen in test serum.
- Primary antibody (targeted against the serum antigen) is labelled with the enzyme.

Steps	Explanation
Step 1	Wells of microtiter plate are empty, not pre-coated with Ag or Ab.
Step-2	Test serum (containing antigen) is added into the wells. Antigen becomes attached to the solid phase by passive adsorption.
Step-3	After washing, the enzyme-labelled primary antibodies (raised in rabbits) are added.
Step-4	After washing, a substrate- chromogen system is added and colour is measured.

Direct ELISA

- Well + Ag (test serum) + primary Ab-Enzyme + substrate- chromogen → Colour change.



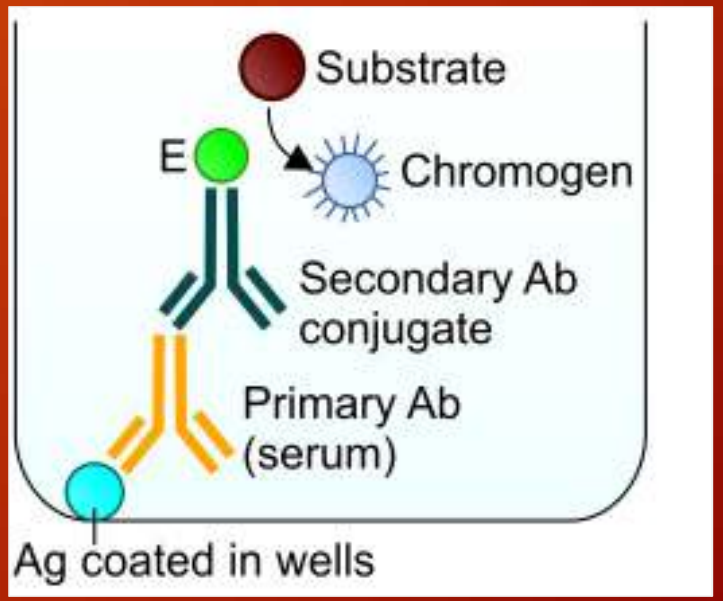
Indirect ELISA – Antibody detection

- It differs from the direct ELISA in that the secondary antibody (Ab targeted to Fc region of any human Ig) is labelled with enzyme instead of primary antibody.

Steps	Explanation
Step 1	Solid phase of the wells of microtiter plates are pre-coated with the Ag.
Step-2	Test serum (containing primary Ab specific to the Ag) is added to the wells. Ab gets attached to the Ag coated on the well.
Step-3	After washing, enzyme-labelled secondary Ab (anti-human immunoglobulin) is added.
Step-4	After washing, a substrate-chromogen system is added and colour is developed.

Indirect ELISA

- Wells are coated with Ag + primary Ab (test serum) + secondary Ab-Enzyme + substrate- chromogen → development of color.



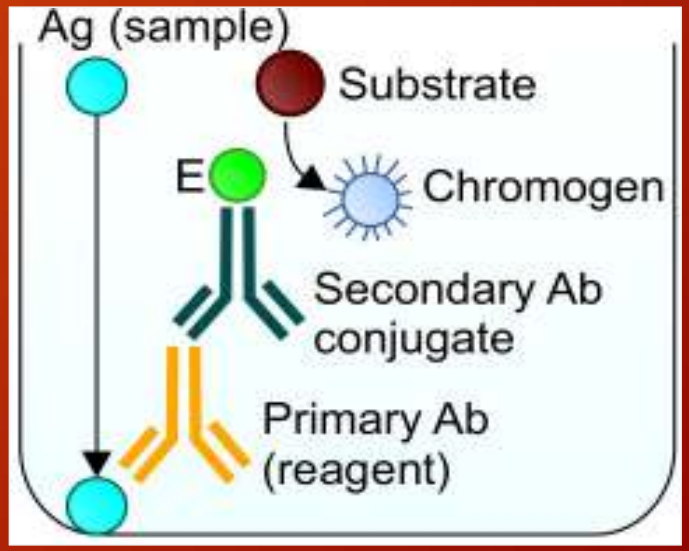
Indirect ELISA – Antigen detection

- Wells are empty, not pre-coated with Ag or Ab.

Steps	Explanation
Step 1	Test antigen (serum) is added to the well. The Ag gets absorbed onto the well.
Step-2	Primary antibody raised in rabbits (reagent) is added. The Ag binds to the primary antibody.
Step-3	After washing, enzyme-labelled secondary Ab (anti-rabbit Ab) is added.
Step-4	After washing, a substrate- chromogen system is added and colour is developed.

Indirect ELISA

- Ag (test serum) added, gets adsorbed to well + primary Ab + secondary Ab-Enzyme + substrate- chromogen → development of color.



Sandwich ELISA

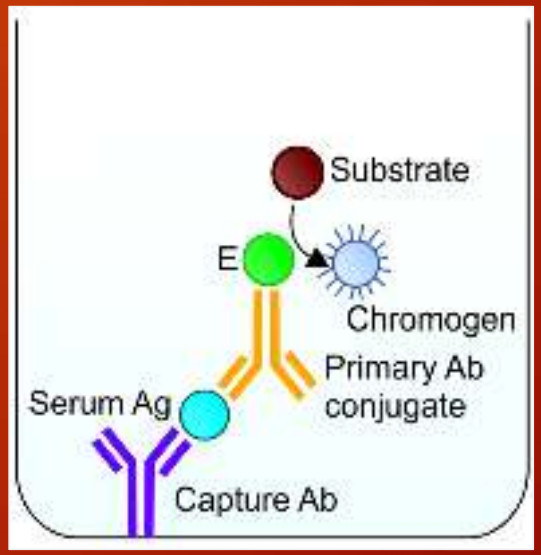
- Detects the antigen in test serum.
- So named because the antigen gets sandwiched between a capture antibody and a detector antibody.
- There are two types of sandwich ELISA:
  - Direct - detector antibody is a primary antibody.
  - Indirect - detector antibody is a secondary antibody.

Direct sandwich ELISA

Steps	Explanation
Step 1	Microtiter well is pre-coated with the capture antibody (monoclonal Ab raised in rabbit) targeted against the test antigen.
Step-2	Test serum (containing antigen) is added to the wells. Ag gets attached to the capture antibody coated on the well.
Step-3	After washing, an enzyme labelled primary ‘detector antibody’ specific for the antigen is added. The detector antibody can be same as the capture antibody.
Step-4	After washing, a substrate-chromogen system is added and colour is developed.

Direct sandwich ELISA

- Wells coated with capture Ab + Ag (test serum) + primary Ab-enzyme + substrate- chromogen → colour

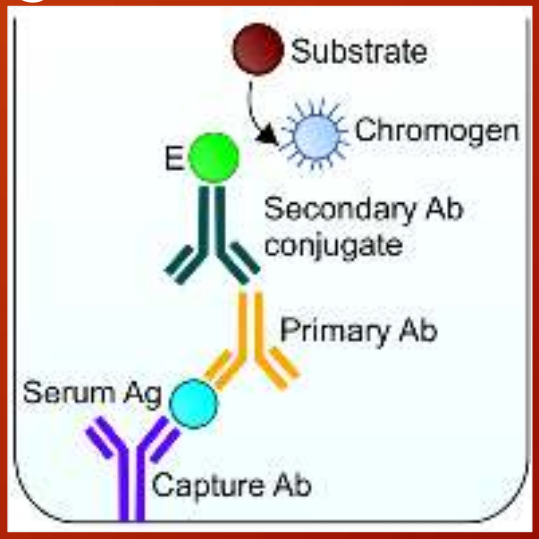


Indirect sandwich ELISA

- Primary antibody and the capture antibody belong to different species. More so, the primary antibody is not labelled with enzyme.
- Another enzyme-labelled secondary antibody targeted against the primary antibody is added.
- More specific than direct sandwich ELISA.

Indirect sandwich ELISA

- Wells coated with capture Ab + Ag (test serum) + primary Ab + secondary Ab- enzyme + substrate- chromogen → color.



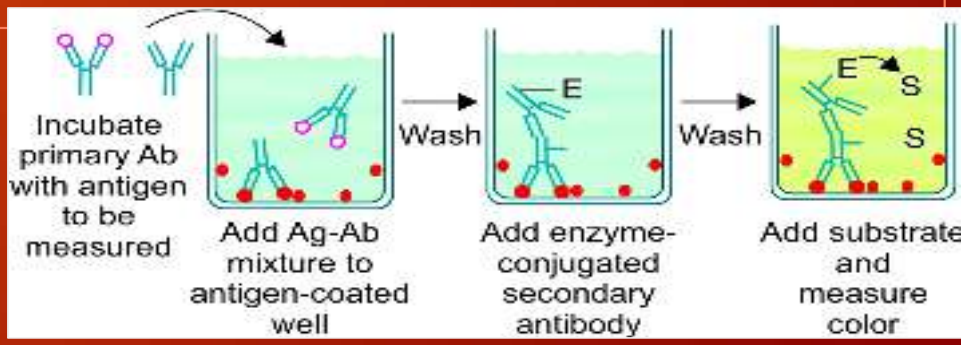
Competitive ELISA

- Example: Indirect competitive ELISA format used for antigen detection.

Steps	Explanation
Step 1	Primary antibody is first incubated in a solution with a serum sample containing the test antigen.
Step-2	Antigen-antibody mixture is then added to the microtiter well pre-coated with the same type of antigen.
Step-3	Free antibodies bind to the antigen coated on the well. More the test antigens present in the sample, lesser free antibodies will be available to bind to the antigens coated onto well.

Competitive ELISA

Steps	Explanation
Step-4	After washing (to remove free antibodies and antigens), enzyme-conjugated secondary antibody is added.
Step-5-	After washing, a substrate- chromogen system is added and colour is developed. Intensity of the colour is ***inversely proportional*** to the amount of antigen present in the test serum.



Competitive ELISA

- Can also be used for the detection of antibody in serum.
- Different formats are available such as direct, indirect and sandwich formats.

Advantage of ELISA

- Method of choice for detection of antigens/antibodies in serum - in big laboratories as large number of samples can be tested together using the 96 well microtiter plate.
- Economical, takes 2-3 hours for performing the assay.
- Most sensitive immunoassay - commonly used for performing screening test at blood banks and tertiary care sites.
- Specificity used to be low - But now, with use of more purified recombinant and synthetic antigens, and monoclonal antibodies, ELISA has become more specific.

Disadvantage of ELISA

- Small laboratories with less sample load - ELISA is less preferred than rapid tests as the later can be done on individual samples.
- More time (2-3 hours) compared to rapid tests which take 10-20 minutes.
- Needs expensive equipments such as ELISA washer and reader.

Applications of ELISA

- **ELISA used for antigen detection** -hepatitis B [hepatitis B surface antigen (HBsAg) and pre-core antigen (HBeAg)], NS1 antigen for dengue etc.

- **ELISA can also be used for antibody detection** against hepatitis B, hepatitis C, HIV, dengue, EBV, HSV, toxoplasmosis, leishmaniasis, etc.

IMMUNOFLUORESCENCE ASSAY

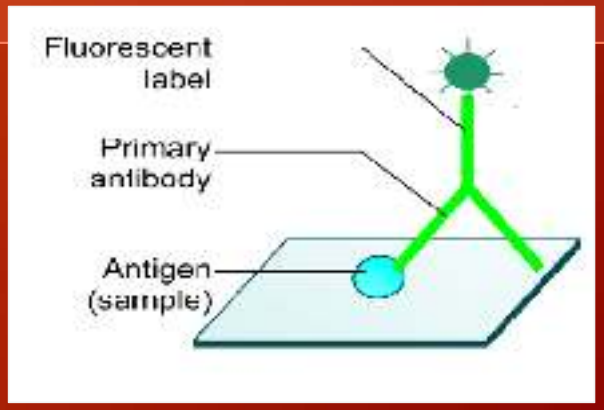
- Technique similar to ELISA, but differs by some important features:
  - Fluorescent dye is used instead of enzyme for labelling of antibody.
  - Detects cell surface antigens or antibodies bound to cell surface antigens, unlike ELISA which detects free antigen or antibody.

IMMUNOFLUORESCENCE ASSAY - Principle

- Fluorescence refers to absorbing high energy-shorter wavelength ultraviolet light rays by the fluorescent compounds and in turn emitting visible light rays with a low energy-longer wavelength.
- Fluorescent dye (FITC) is used to conjugate the antibody and such labelled antibody can be used to detect the antigens or antigen-antibody complex on the cell surface.
- **Types of Immunofluorescence Assays :** *Direct and Indirect*.

Direct Immunofluorescence Assay

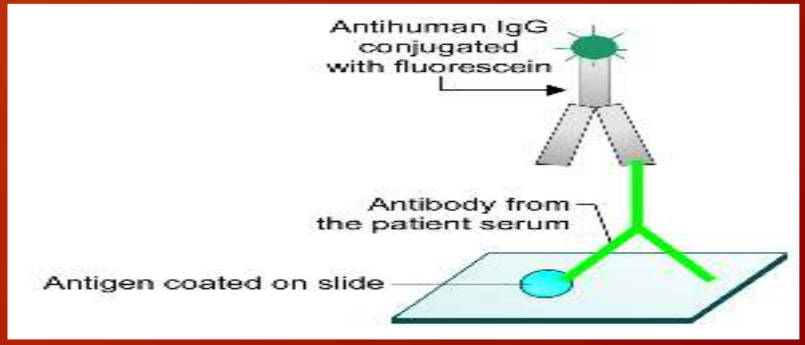
Steps	Explanation
Step-1	Sample containing cells carrying surface antigens is smeared on a slide.
Step-2	Primary antibody specific to the antigen, tagged with fluorescent dye is added.
Step-3	Slide is washed to remove the unbound antibodies and then viewed under a fluorescence microscope.



Indirect Immunofluorescence Assay

- Detects antibodies in sample. Slides smeared with cells carrying known antigens are commercially available.

Steps	Explanation
Step-1	Test serum containing primary antibody is added to the slide.
Step-2	Slide is washed to remove the unbound antibodies. A secondary antibody (anti human antibody conjugated with fluorescent dye) is added.
Step-3	Slide is washed and then viewed under a fluorescence microscope



Applications

- Detection of autoantibodies (e.g. antinuclear antibody) in autoimmune diseases.
- Detecting microbial antigens, e.g. Rabies antigen in corneal smear
- Detection of viral antigens in cell lines inoculated with the specimens


THANK
YOU!

Notes

61

COMPLEMENT FIXATION TEST

61.1 INTRODUCTION

Jules Bordet's pioneering research made clear the exact manner by which serums and antiserums act to destroy bacteria and foreign blood cells in the body, thus explaining how human and animal bodies defend themselves against the invasion of foreign elements. Bordet was also responsible for developing complement fixation tests, which made possible the early detection of many disease-causing bacteria in human and animal blood.

OBJECTIVES

After reading this chapter, the student will be able to:

- describe the term Complement
- explain the principle of Complement Fixation Test
- describe steps involved in the Complement Fixation Test
- enlist the uses of Complement Fixation Test
- describe the modifications of Complement Fixation Test

61.2 COMPLEMENT FIXATION TEST

In 1894, Richard Pfeiffer, a German scientist, had discovered that when cholera bacteria were injected into the peritoneum of a guinea pig immunized against the infection, the pig would rapidly die. This bacteriolysis, Bordet discovered, did not occur when the bacteria was injected into a non-immunized guinea pig, but did so when the same animal received the antiserum from an immunized animal. Moreover, the bacteriolysis did not take place when the bacteria and the antiserum were mixed in a test tube unless fresh antiserum was used. However,

when Bordet heated the antiserum to 55 degrees centigrade, it lost its power to kill bacteria. Finding that he could restore the bacteriolytic power of the antiserum if he added a little fresh serum from a non-immunized animal, Bordet concluded that the bacteria-killing phenomenon was due to the combined action of two distinct substances: an antibody in the antiserum, which specifically acted against a particular kind of bacterium; and a non-specific substance, sensitive to heat, found in all animal serums, which Bordet called "alexine" (later named "complement").

In a series of experiments conducted later, Bordet also learned that injecting red blood cells from one animal species (rabbit cells in the initial experiments) into another species (guinea pigs) caused the serum of the second species to quickly destroy the red cells of the first. And although the serum lost its power to kill the red cells when heated to 55degrees centigrade, its potency was restored when alexine (or complement) was added. It became apparent to Bordet that haemolytic (red cell destroying) serums acted exactly as bacteriolytic serums; thus, he had uncovered the basic mechanism by which animal bodies defend or immunize themselves against the invasion of foreign elements. Eventually, Bordet and his colleagues found a way to implement their discoveries. They determined that alexine was bound or fixed to red blood cells or to bacteria during the immunizing process. When red cells were added to a normal serum mixed with a specific form of bacteria in a test tube, the bacteria remained active while the red cells were destroyed through the fixation of alexine. However, when serum containing the antibody specific to the bacteria was destroyed, the alexine and the solution separated into a layer of clear serum overlaying the intact red cells. Hence, it was possible to visually determine the presence of bacteria in a patient's blood serum. This process became known as a complement fixation test. Bordet and his associates applied these findings to various other infections, like typhoid fever, carbuncle, and hog cholera. August VonWasserman eventually used a form of the test (later known as the Wasserman test) to determine the presence of syphilis bacteria in the human blood**.**

The complement fixation test (CFT) was extensively used in syphilis serology after being introduced by Wasserman in 1909. It took a number of decades before the CFT was adapted for routine use in virology.

CFT meet the following criteria

- it is convenient and rapid to perform
- the demand on equipment and reagents is small
- a large variety of test antigens are readily available.

However, there is now a trend to replace the CFT with more direct, sensitive and rapid techniques, such as RIAs and EIAs. Although CFT is considered to

be a relatively simple test, it is a very exacting procedure because variables are involved

Guinea pig is the commonest source of fresh complement. The serum should be collected from guinea pig just before the test because complement is easily destroyed by heat. However, complement can be preserved either by lipophilizing, freezing or by adding preservatives. Preserved complement is also obtained from commercial sources. Complement should be titrated for its haemolytic activity. One unit or minimum haemolytic dose (MHD) is the highest dilution of the guinea pig serum that lyses one unit volume of washed sheep red blood cells in the presence of excess of haemolysin (amboceptor) in either 30 or 60 minutes, at 37C. Physiological saline with added magnesium and calcium ions is used as the diluent for titration and CFT.

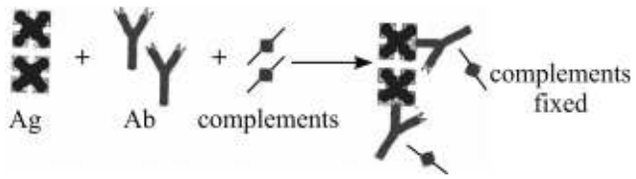


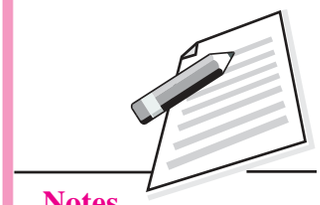
Fig. 61.1: Complement fixation

Complement fixation test consists of a test system and an indicator system, both of which can activate complement. When used to detect the presence of an antibody the test system is formed by the patient's serum and a known antigen. The indicator system is formed by sheep red blood cells coated with rabbit antibody to sheep red cells (amboceptors). The sheep red blood cells will lyse in the presence of complement.

- Sheep red cells: 5% suspension of washed sheep red blood cells should be used.
- Haemolysin (amboceptors): it is an antibody to sheep red cells which raised in rabbit. It should also be titrated for haemolytic activity. The MHD of the amboceptor is the highest dilution of the inactivated an amboceptor, which lyses one unit volume of sheep red blood cells in the presence of excess complement in 30 or 60 minutes at 37°C.

CFT consists of two steps:

Step 1: a known antigen and inactivated patient's serum are incubated with a standardized, limited amount of complement. If the serum contains specific, complement activating antibody the complement will be activated or fixed by the antigen-antibody complex. However, if there is no antibody in the patient's serum, there will be no formation of antigen-antibody complex, and therefore complement will not be fixed. But will remain free.



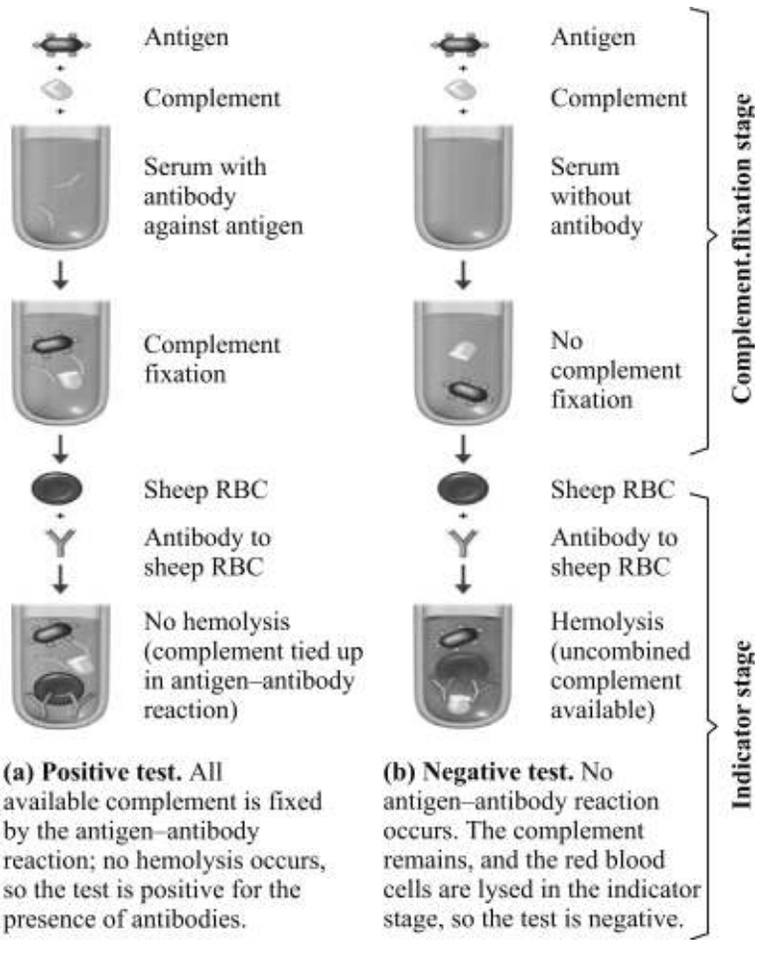


Fig. 61.2: Complement Fixation Test

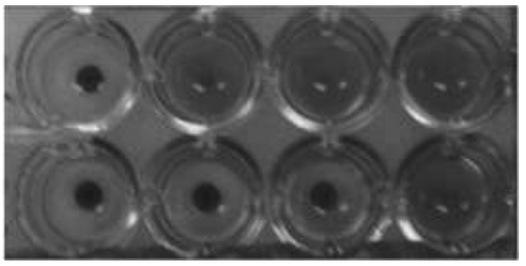
Step 2: The second step detects whether complement has been utilized in the first step or not. This is done by adding the indicator system. If the complement is fixed in the first step owing to the presence of antibody there will be no complement left to fix to the indicator system. However, if there is antibody in the patient's serum, there will be no antigen-antibody complex, and therefore, complement will be present free or unfixed in the mixture. This unfixed complement will now react with the antibody- coated sheep red blood cells to bring about their lysis. Thus, no lysis of sheep red blood cells (positive CFT) indicates the presence of antibody in the presence of antibody in the test serum, while lysis of sheep red blood cells (Negative CFT) indicates the absence of antibody in the serum.

Controls should be used along with the test to ensure that

(a) Antigen and serum are not anti complimentary

(b) The appropriate amount of complement is used and

(c) The sheep red blood cells do not undergo autolysis.



Complement Fixation Test in Microtiter Plate, rows 1 and 2 exhibit complement fixation obtained with acute and convalescent phase serum specimens, respectively. (2-fold serum dilutions were used) The observed 4-fold increase is significant and indicates infection.

Advantages of CFT

1. Ability to screen against a large number of viral and bacterial infections at the same time.
2. Economical.

Disadvantages of CFT

1. Not sensitive - cannot be used for immunity screening
2. Time consuming and labor intensive
3. Often non-specific e.g. cross-reactivity between HSV and VZV

Modifications of complement fixation test

(a) **Indirect complement fixation test:** This modification is used when serums which don't fix guinea pig complement is to be tested. Here, the test is set up in duplicate. After step 1, standard antiserum to antigen which is known to fix complement is added to one set. If antibodies were not present in the test serum then the antigen would react with the standard antiserum fixing the complement. On the other hand if antibodies are present in the test serum the antigen would be utilized in the first step. So, no reaction would occur between the standard antiserum and the antigen and therefore no fixation of complement would cause lysis of sheep red blood cells. Thus in this case haemolysis indicates a positive result.

(b) **Congulatinating complement absorption test**: Here horse complement which is non-haemolytic is used. The indicator system used is sensitized sheep red blood cells mixed with bovine serum. Bovine serum contains

a beta globulin called conglutinin would also combine with this complement causing agglutination (conglutination) of the sheep red blood cells, indicating a negative result.

(c) Immune adherence: When some bacteria (such as vibrio cholera or treponemapallidum) combine with their specific antibody in the presence of complement and some particles such as erythrocytes or platelets, they adhere to the erythrocytes or platelets. This is called immune adherence.

(d) Immobilisation test: Here antigen is incubated with patient's serum in presence of complement. If specific antibody is present it would immobilize the antigen. Eg.Treponema palladium immobilization test, considered gold standard for the serodiagnosis of syphilis.

(e) Cytolytic tests: The incubation of a live bacterium with its specific antibody in the presence of complement leads to the lysis of the bacteria cells. This is the basis of vibriocidal antibody test used to measure anti-cholera antibodies.

INTEXT QUESTION 61.1

1. The complement fixation test was initially used in ..... serology
2. The commonest source of fresh complement is .....
3. The highest dilution of guinea pig serum that lyses red blood cells is called .....
4. ..... is the antibody to sheep red cells used in complement fixation test
5. ..... is used in coagulatinating complement absorption test
6. When bacilli combine with specific antibody in the presence of complement is called .....
7. Gold standard serodiagnosis of syphilis is by ..... test
8. ..... test is commonly used in diagnosis of cholera

WHAT HAVE YOU LEARNT

- CFT was developed by Jules Bordet. Complement fixation test consists of a test system and an indicator system, both of which can activate complement. When used to detect the presence of an antibody the test system is formed by the patient's serum and a known antigen. The indicator system is formed

by sheep red blood cells coated with rabbit antibody to sheep red cells (amboceptors). The sheep red blood cells will lyse in the presence of complement. There exits modification of complement fixation test- Indirect complement fixation test, Congulatinating complement absorption test, Immune adherence, Immobilisation test, Cytolytic tests.

TERMINAL QUESTIONS

1. What do you understand by the term complement?
2. Describe in brief CFT.
3. Give the advantages anf the limitations of CFT
4. Explain in brief various modifications of complement fixation test with proper examples.
5. Explain the term amboceptor.
6. Mention the advantages and disadvantges of CFT.
7. Enlist the modifications of complement fixation test

ANSWERS TO INTEXT QUESTIONS

61.1

1. Syphillis
2. Guinea Pig
3. Minimum Haemolytic Dose
4. Amboceptors
5. Horse Complement
6. Immune Adherences
7. Immobilization
8. Cytolytic

Difference Between Humoral and Cell Mediated Immunity

September 28, 2017 • by Lakna • 6 min read

Main Difference – Humoral Immunity vs Cell mediated immunity

Humoral immunity and cell mediated immunity are two types of adaptive immunity. Adaptive immunity generates an antigen-specific immune response. During adaptive immunity, the antigen is first recognized through receptors of the lymphocytes, and immune cell clones are produced to attack that particular antigen. Humoral immunity is triggered by B cells while cell mediated immunity is triggered by T cells. The **main difference** between humoral and cell mediated immunity is that **antigen-specific antibodies are produced in humoral immunity whereas antibodies are not produced in cell mediated immunity**. Instead, T cells destroy the infected cells by inducing apoptosis.

Key Areas Covered

Key Terms: Antibodies, Cell mediated immunity, Cytotoxic T cells, Extracellular Pathogens, Helper T Cells, Humoral Immunity, Intracellular Pathogens, Opsonization, Phagocytosis, Plasma B Cells

What is Humoral Immunity

Humoral immunity is the immunity generated by circulating antibodies. It is a component of adaptive immunity, which generates specific immune responses to a particular foreign material. The extracellular spaces of the body are

protected by humoral immunity. Most pathogens that invade the body multiply in the extracellular spaces. Intracellular pathogens move from one cell to another through the extracellular space. Therefore, extracellular space is an important place to destroy pathogens. Antibodies are produced and secreted by plasma B cells. Typically, the activation of B cells occurs in T helper cells.

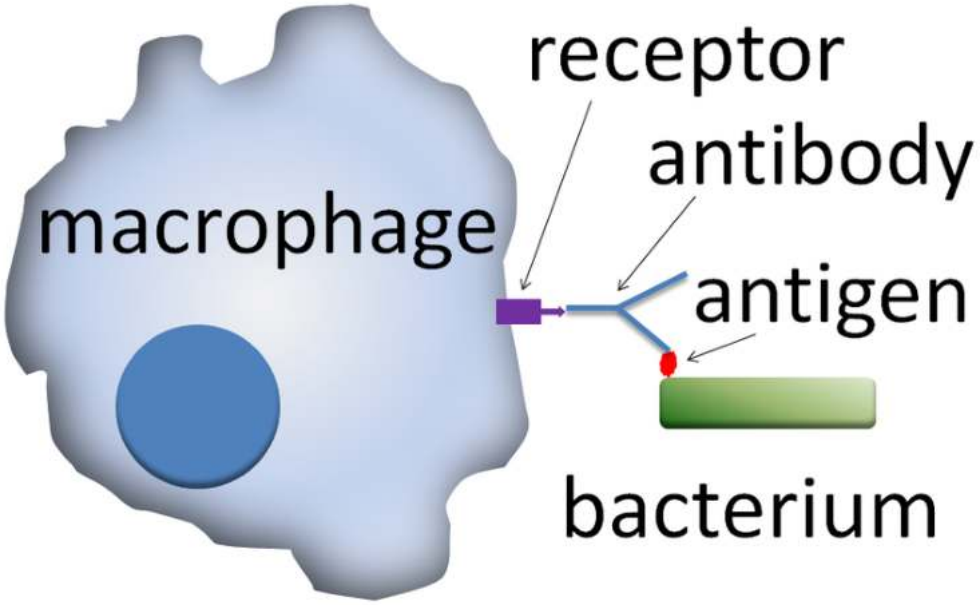


Figure 1: Opsonization

Antibodies destroy pathogens in three ways. They bind to the specific molecules on the surface of the pathogen, neutralizing the pathogen. This neutralization prevents the entering of the pathogen to the cells. It is also important to prevent bacterial toxins. The antibody-caught pathogens are subjected to **phagocytosis** by macrophages and other cells. This process is called **opsonization**. The binding of antibodies to the pathogens activates the complement system. The complement proteins bind to the antibody-bound pathogens and recruit phagocytic cells. The opsonization is shown in *figure 1*.

What is Cell Mediated Immunity

Cell mediated immunity is the immunity mediated by antigen-specific T cells. T cells are produced in the bone marrow and are matured in the thymus. After they enter the bloodstream, T cells occur can be found in the blood as well as in lymphoid tissue. The antigens should be presented on the surface of the antigen-presenting cells (APCs) along with the major histocompatibility complexes (MHC). Once T cells encounter an antigen, they proliferate and differentiate into armed effector cells. The cytotoxic T cells destroy the infected cells by inducing apoptosis. T helper cells stimulate plasma B cells to produce antibodies.

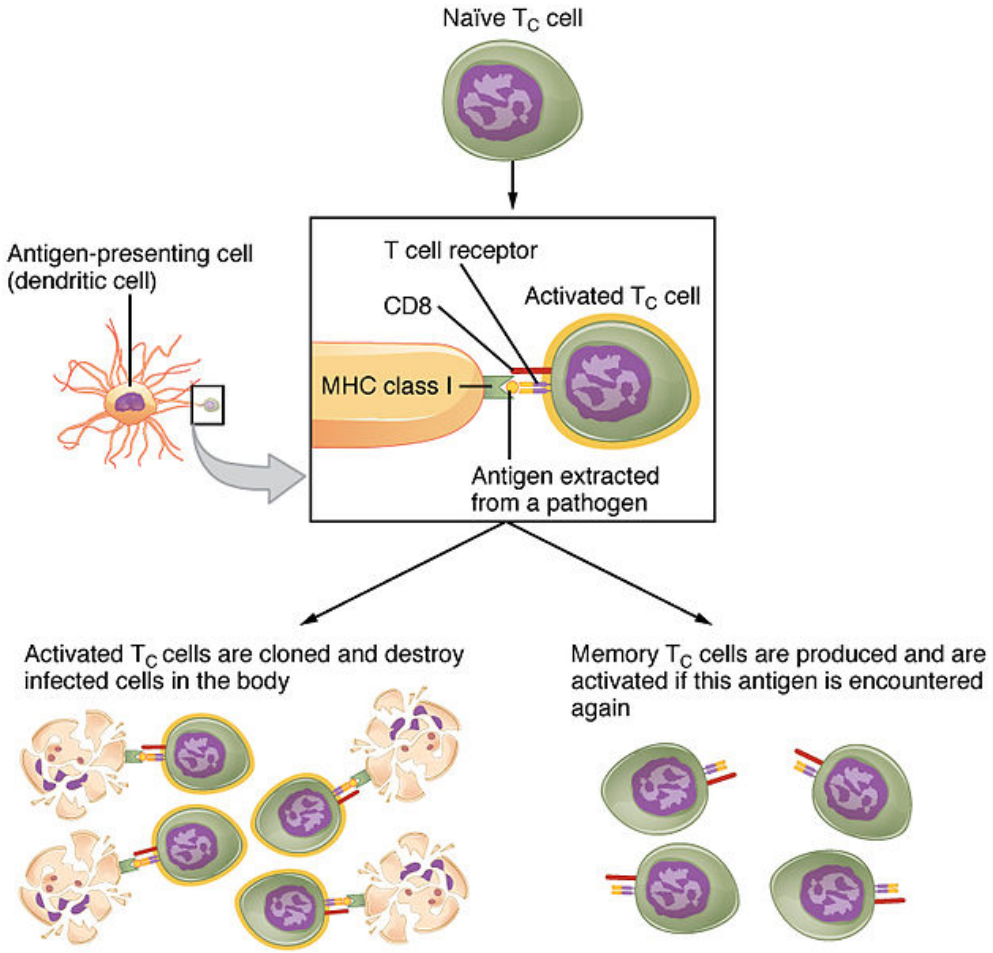


Figure 2: Cell Mediated Immunity

The IgG and IgM are the main two types of antibodies produced by T helper cells in response to plasma B cells. The memory T cells are differentiated T cells, but their action requires the activation by the specific antigen. The major characteristic feature of the cell mediated immunity is that it destroys intracellular pathogens. The cell mediated immunity is shown in *figure 2*.

Similarities Between Humoral Immunity and Cell Mediated Immunity

- Humoral immunity and cell mediated immunity are two types of adaptive immunity.
- Both humoral immunity and cell mediated immunity produce a specific immune response to a particular pathogen.

Difference Between Humoral and Cell Mediated Immunity Definition

Humoral Immunity: Humoral immunity refers to a component of the adaptive immunity where B cells secrete antibodies, which circulate in the blood as a soluble protein.

Cell Mediated Immunity: Cell mediated immunity refers to the other component of the adaptive immunity, which is mediated by the activated, antigen-specific T cells.

Main Cells

Humoral Immunity: The humoral immunity is mediated by B cells.

Cell Mediated Immunity: The cell mediated immunity is mediated by T cells.

Cell Types

Humoral Immunity: Humoral immunity is mediated by T cells, B cells, and macrophages.

Cell Mediated Immunity: Cell mediated immunity is mediated by helper T cells, cytotoxic T cells, natural killer cells, and macrophages.

Action

Humoral Immunity: The humoral immunity acts on the extracellular microbes and their toxins.

Cell Mediated Immunity: The cell mediated immunity acts on intracellular microbes such as viruses, bacteria, and parasites and tumor cells.

Receptors

Humoral Immunity: The BCR receptors are involved in the humoral immunity.

Cell Mediated Immunity: The TCR receptors are involved in the cell mediated immunity.

Accessory Surface Molecules

Humoral Immunity: The Igα, Igβ, CD40, CD21, and Fc receptors are the accessory receptors of the humoral immunity.

Cell Mediated Immunity: The CD2, CD3, CD4, CD8, CD28, and integrins are the accessory receptors of the cell mediated immunity.

Role of MHC Molecules

Humoral Immunity: The unprocessed antigens are recognized by the humoral immunity.

Cell Mediated Immunity: The antigens are processed and presented by MHC complexes in the cell mediated immunity.

Secretion

Humoral Immunity: The plasma B cells secrete antibodies in the humoral immunity.

Cell Mediated Immunity: The T cells secrete cytokines.

Onset

Humoral Immunity: The humoral immune response is rapid.

Cell Mediated Immunity: The cell-mediated immune response is a delayed type of hypersensitivity.

Tumor Cells and Transplants

Humoral Immunity: The humoral immunity does not act on the tumor cells and transplants.

Cell Mediated Immunity: The cell mediated immunity acts on tumor cells and transplants.

Conclusion

Humoral immunity and cell mediated immunity are two types of adaptive immunity in which a specific immune response is produced for a particular pathogen. Antibodies are produced by the plasma T cells in the humoral immunity. In cell mediated immunity, T cells induce the apoptosis of the infected cells. Humoral immunity destroys the extracellular pathogens whereas cell mediated immunity destroys the intracellular pathogens. This is the difference between humoral and cell mediated immunity.

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About the Author: Lakna

Lakna, a graduate in Molecular Biology & Biochemistry, is a Molecular Biologist and has a broad and keen interest in the discovery of nature related things

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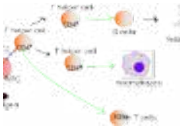
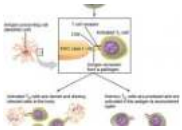
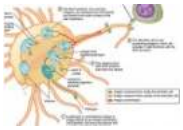


Difference Between Lymphatic and Immune System

Difference Between Trachea and Esophagus



Difference Between Humoral and Cell Mediated Immunity



Difference Between Lymphocytes and Phagocytes

Difference Between Ganglia and Nuclei

Chapter 2

Immobilization of Enzymes: A Literature Survey

Beatriz Brena, Paula González-Pombo, and Francisco Batista-Viera

Abstract

The term immobilized enzymes refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously."

Immobilized enzymes are currently the subject of considerable interest because of their advantages over soluble enzymes. In addition to their use in industrial processes, the immobilization techniques are the basis for making a number of biotechnology products with application in diagnostics, bioaffinity chromatography, and biosensors. At the beginning, only immobilized single enzymes were used, after 1970s more complex systems including two-enzyme reactions with cofactor regeneration and living cells were developed.

The enzymes can be attached to the support by interactions ranging from reversible physical adsorption and ionic linkages to stable covalent bonds. Although the choice of the most appropriate immobilization technique depends on the nature of the enzyme and the carrier, in the last years the immobilization technology has increasingly become a matter of rational design.

As a consequence of enzyme immobilization, some properties such as catalytic activity or thermal stability become altered. These effects have been demonstrated and exploited. The concept of stabilization has been an important driving force for immobilizing enzymes. Moreover, true stabilization at the molecular level has been demonstrated, e.g., proteins immobilized through multipoint covalent binding.

Key words Immobilized enzymes, Bioaffinity chromatography, Biosensors, Enzyme stabilization, Immobilization methods

1 Background

Enzymes are biological catalysts that promote the transformation of chemical species in living systems. These molecules, consisting of thousands of atoms in precise arrangements, are able to catalyze the multitude of different chemical reactions occurring in biological cells. Their role in biological processes, in health and disease, has been extensively investigated. They have also been a key component in many ancient human activities, especially food processing, well before their nature or function was known [1].

Jose M. Guisan (ed.), *Immobilization of Enzymes and Cells: Third Edition*, Methods in Molecular Biology, vol. 1051,
DOI 10.1007/978-1-62703-550-7_2,

Table 1
Technological properties of immobilized enzyme systems [3]

Advantages	Disadvantages
Catalyst reuse	Loss or reduction in activity
Easier reactor operation	Diffusional limitation
Easier product separation	Additional cost
Wider choice of reactor	

Enzymes have the ability to catalyze reactions under very mild conditions with a very high degree of substrate specificity, thus decreasing the formation of by-products. Among the reactions catalyzed are a number of very complex chemical transformations between biological macromolecules, which are not accessible to ordinary methods of organic chemistry. This makes them very interesting for biotechnological use. At the beginning of the twentieth century, enzymes were shown to be responsible for fermentation processes and their structure and chemical composition started to come under scrutiny [2]. The resulting knowledge leads to the widespread technological use of biological catalysts in a variety of other fields such as textile, pharmaceutical, and chemical industries. However, most enzymes are relatively unstable, their costs of isolation are still high, and it is technically very difficult to recover the active enzyme, when used in solution, from the reaction mixture after use.

Enzymes can catalyze reactions in different states: as individual molecules in solution, in aggregates with other entities, and as attached to surfaces. The attached or "immobilized" state has been of particular interest to those wishing to exploit them for technical purposes. The term *immobilized enzymes* refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" [3]. The introduction of immobilized catalysts has, in some cases, greatly improved both the technical performance of the industrial processes and their economy (Table 1).

The first industrial use of immobilized enzymes was reported in 1966 by Chibata and coworkers, who developed the immobilization of *Aspergillus oryzae* aminoacylase for the resolution of synthetic racemic D-L amino acids [4]. Other major applications of immobilized enzymes are the industrial production of sugars, amino acids, and pharmaceuticals (Table 2) [5]. In some industrial processes, whole microbial cells containing the desired enzyme are immobilized and used as catalysts [6].

Table 2
Major products obtained using immobilized enzymes [3, 5]

Enzyme	Product
Glucose isomerase	High-fructose corn syrup
Amino acid acylase	Amino acid production
Penicillin acylase	Semi-synthetic penicillins
Nitrile hydratase	Acrylamide
β-Galactosidase	Hydrolyzed lactose (whey)

Aside from the application in industrial processes, the immobilization techniques are the basis for making a number of biotechnology products with application in diagnostics, bioaffinity chromatography, and biosensors [7, 8]. Therapeutic applications are also foreseen, such as the use of enzymes in extra-corporeal shunts [9].

In the past four decades, immobilization technology has developed rapidly and has increasingly become a matter of rational design but there is still the need for further development [10]. Extension of the use of immobilized enzymes to other practical processes will require both new methodologies and better understanding of those used at present.

2 History of Enzyme Immobilization

It is possible to visualize four steps in the development of immobilized biocatalysts (Table 3). In the first step at the beginning of the nineteenth century, immobilized microorganisms were being employed industrially on an empirical basis. This was the case of the microbial production of vinegar by letting alcohol-containing solutions trickle over wood shavings overgrown with bacteria, and that of the trickling filter or percolating process for waste water clarification [11].

The modern history of enzyme immobilization goes back to the late 1940s, but much of the early work was largely ignored for biochemists since it was published in Journals of other disciplines [12]. Since the pioneering work on immobilized enzymes in the early 1960s, when the basis of the present technologies was developed, more than 10,000 papers and patents have been published on this subject, indicating the considerable interest of the scientific community and industry in this field [4]. In the second step, only immobilized single enzymes were used but by the 1970s more complex systems, including two-enzyme reactions with cofactor

Table 3
Steps in the development of immobilized enzymes [11, 14]

Step	Date	Use
First	1815	Empirical use in processes such as acetic acid and waste water treatment.
Second	1960s	Single enzyme immobilization: production of L-amino acids, isomerization of glucose, etc.
Third	1985–1995	Multiple enzyme immobilization including cofactor regeneration and cell immobilization. Example: production of L-amino acids from keto-acids in membrane reactors.
Fourth	1995 to present	Ever-expanding multidisciplinary developments and applications to different fields of research and industry.

regeneration and living cells were developed [13]. As an example of the latter we can mention the production L-amino acids from α-keto acids by stereoselective reductive amination with L-amino acid dehydrogenase. The process involves the consumption of NADH and regeneration of the coenzyme by coupling the amination with the enzymatic oxidation of formic acid to carbon dioxide with concomitant reduction of NAD+ to NADH, in the reaction catalyzed by the second enzyme, formate dehydrogenase. More recently, in the last few decades, immobilized enzyme technology has become a multidisciplinary field of research with applications to clinical, industrial and environmental samples [14].

The major components of an immobilized enzyme system are: the enzyme, the support and the mode of attachment of the enzyme to the matrix. The term solid-phase, solid support, support, carrier, and matrix are used synonymously.

3 Choice of Supports

The characteristics of the matrix are of paramount importance in determining the performance of the immobilized enzyme system. Ideal support properties include physical resistance to compression, hydrophilicity, inertness towards enzymes, ease of derivatization, bio-compatibility, resistance to microbial attack, and availability at low cost [12–15]. However, even though immobilization on solid supports is an established technology, there are still no general rules for selecting the best support for a given application.

Supports can be classified as inorganic and organic, according to their chemical composition (Table 4). The organic supports can be subdivided into natural and synthetic polymers [16].

Table 4
Classification of supports

Organic
Natural polymers
• Polysaccharides: cellulose, dextrans, agar, agarose, chitin, alginate
• Proteins: collagen, albumin
• Carbon
Synthetic polymers
• Polystyrene
• Other polymers: polyacrylate, polymethacrylates, polyacrylamide, polyamides, vinyl and allyl-polymers
Inorganic
Natural minerals
Bentonite, silica
Processed materials
Glass (non-porous and controlled pore), metals, controlled pore metal oxides

The physical characteristics of the matrices (such as mean particle diameter, swelling behavior, mechanical strength, compression behavior) will be of major importance for the performance of the immobilized systems and determine the type of reactor used under technical conditions (i.e., stirred tank, fluidized, fixed beds). In particular, pore parameters and particle size determine the total surface area and thus critically affect the capacity for binding of enzymes. Nonporous supports show few diffusional limitations but have a low loading capacity. Therefore, porous supports are in general preferred because the high surface area allows a higher enzyme loading and the immobilized enzyme is more protected from the environment. Porous supports should have a controlled pore distribution in order to optimize capacity and flow properties. In spite of the many advantages of inorganic carriers (e.g., high stability against physical, chemical, and microbial degradation), most of the industrial applications are performed with organic matrices. The hydrophilic character is one of the most important factors determining the level of activity of an immobilized enzyme [17].

Agarose is an excellent matrix which has been extensively used. In addition to its high porosity which leads to a high capacity for proteins, some other advantages of using agarose are hydrophilic character, ease of derivatization, absence of charged groups (which prevents nonspecific adsorption of substrate and products), and

commercial availability. However, an important limitation of agarose and other porous supports is the high cost. An approach to avoid this problem is the use of reversible methods of immobilization that allow matrix regeneration and reuse.

In turn, macroporous acrylic polymers such as Eupergit® C (Röhm, Darmstadt, Germany) and Sepabeads® EC (Resindion, Milan, Italy), are suitable carriers for covalent immobilization of enzymes for industrial applications, and are amongst the most extensively studied matrixes [18–20].

Nanomaterials can serve as excellent support materials for enzyme immobilization, offering ideal characteristics for balancing the key factors that determine the efficiency of biocatalysts: surface area, mass transfer resistance and effective enzyme loading [21, 22]. Nanotechnology has provided a wide variety of alternatives for enzyme immobilization leading to potential applications in biotechnology, immunosensing, and biomedical areas [23]. Recently, enzymes immobilized to nanosized supports such as polymer microspheres, fibers, tubes, as well as various metal and magnetic nanoparticles have been reported [23–25].

4 Methods of Immobilization

In the last decades, thousands of protocols have been reported in the literature [26–29] and various immobilization strategies can be envisioned [30]. The enzymes can be attached to the support by interactions ranging from reversible physical adsorption and ionic linkages to stable covalent bonds. One way of classifying the various approaches to immobilizing enzymes is in two broad categories: irreversible and reversible methods [31] (Fig. 1). The strength

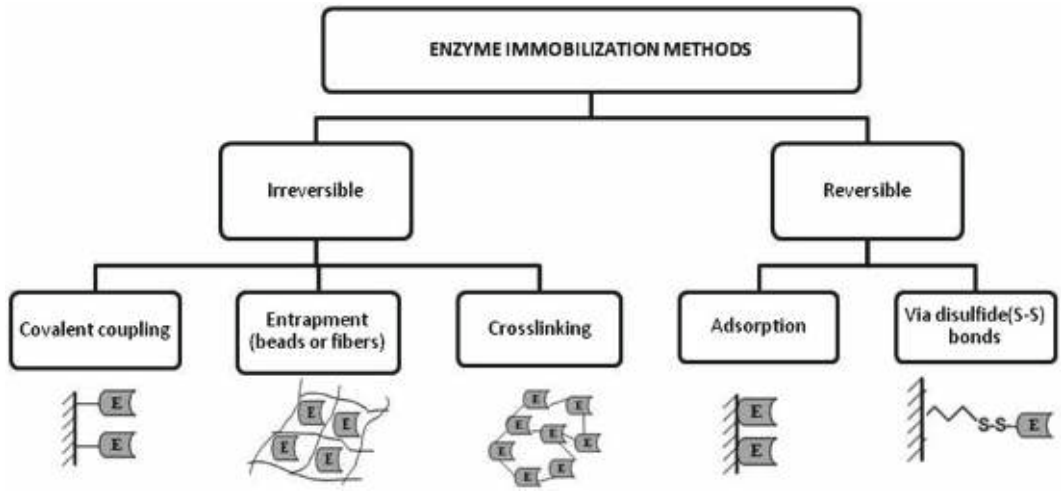


Fig. 1 Schematic representation of the main different methods of enzyme immobilization (*E* enzyme)

Table 5
Advantages and disadvantages of the main enzyme immobilization methods

Methods and binding nature	Advantages	Disadvantages
Physical adsorption Weak bonds: hydrophobic, Van der Waals or ionic interactions.	Simple and cheap Little conformational change of the enzyme	Desorption Nonspecific adsorption
Affinity Affinity bonds between two affinity partners	Simple and oriented immobilization Remarkable selectivity	High cost
Covalent binding Chemical binding between functional groups of the enzyme and support	No enzyme leakage Potential for enzyme stabilization	Matrix and enzyme are not regenerable Major loss of activity
Entrapment Occlusion of an enzyme within a polymeric network	Wide applicability	Mass transfer limitations Enzyme leakage
Cross-linking Enzymes molecules are cross-linked by a functional reactant	Biocatalyst stabilization	Cross-linked biocatalysts are less useful for packed beds. Mass transfer limitations Loss of activity

of the binding is usually inversely related to the ease with which it can be reversed. These two conflicting objectives, stability, and reversibility are difficult to fulfill simultaneously. The traditional approach has been to make the bond as strong as possible and sacrifice reversibility.

In addition, immobilization methods are often classified by the type of chemical reaction used for binding (Table 5). In some cases, enzyme immobilization protocols are also based on the combination of several immobilization methods. For example, an enzyme can be pre-immobilized on beads by adsorption, affinity, or covalent bonds before further entrapment in a porous polymer.

Each immobilization method presents advantages and drawbacks (Table 5). The choice of the most appropriate technique also depends on the nature of the enzyme (biochemical and kinetics properties) and the carrier (chemical characteristics, mechanical properties). So, the interaction between the enzyme and support provides an immobilized enzyme with particular biochemical and physicochemical properties that determine their applicability to specific processes.

5 Methods of Irreversible Enzyme Immobilization

The concept of irreversible immobilization means that once the biocatalyst is attached to the support, it cannot be detached without destroying either the biological activity of the enzyme or the support. The most common procedures of irreversible enzyme immobilization are covalent coupling, entrapment or microencapsulation, and cross-linking (Fig. 1).

5.1 Formation of Covalent Bonds

Immobilization of proteins by methods based on the formation of covalent bonds is among the most widely used. An advantage of these methods is that, because of the stable nature of the bonds formed between enzyme and matrix, the enzyme is not released into the solution upon use. However, in order to achieve high levels of bound activity, the amino acid residues essential for catalytic activity must not be involved in the covalent linkage to the support, and this may prove a difficult requirement to fulfill in some cases. A simple procedure that sometimes improves the activity yield is to carry out the coupling reaction in the presence of substrate analogues [32]. Covalent methods for immobilization are employed when there is a strict requirement for the absence of the enzyme in the product.

A wide variety of reactions have been developed depending on the functional groups available on the matrix [33]. Coupling methods in general can be divided in two main classes: (1) activation of the matrix by addition of a reactive function to a polymer; (2) modification of the polymer backbone to produce an activated group (Tables 6 and 7). The activation processes are generally designed to generate electrophilic groups on the support which in the coupling step react with the strong nucleophiles on the proteins. The basic principles controlling the course of covalent coupling to the matrices are analogous to those used for the chemical modification of proteins. The most frequently used reactions involve the following side chains of the amino acids: lysine (ε-amino group), cysteine (thiol group), aspartic and glutamic acids (carboxylic group).

There are many commercially available supports for immobilization; the best choice in each case requires the consideration of some relevant properties of the catalyst and the intended use. However, it is usually necessary to try more than one approach and then adapt a method to the specific circumstances [34].

The covalent reactions commonly employed give rise to enzymes linked to the support through, e.g., amide, ether, thioether, or carbamate bonds. Therefore, the enzyme is strongly bound to the matrix and in many cases it is also stabilized, which will be discussed later in Subheading 7. However, because of the covalent nature of the bond, the matrix has to be discarded together

Table 6
Covalent coupling methods of enzymes: activation of matrix hydroxyl functions

Activation method	Group that reacts (with activated matrix)	References
Tresyl chloride, sulfonyl chloride Excellent Thiols, amines 0.1–1.0 sulfonyl Chlorides	Thiol, amine	[35]
Cyanogen bromide	Amine	[36]
Bis oxiranes (epoxides)	Thiol, amine	[37]
Epichlorohydrin	Thiol, amine	[37]
Glutaraldehyde	Amine	[37]
Glycidol-Glyoxyl	Amine	[38]
N-Hydroxy-succinimidyl	Amine	[39, 40]

Table 7
Covalent coupling methods of enzymes: modification of the polymer backbone to produce an activated group

Polymer	Group that reacts	Reagent	Activated group produced	Group that reacts (with activated matrix)	References
Cellulose Agarose	Diol	Periodate	Aldehyde	Amine	[41]
Polyacrylamide	Amide	Hydrazine	Hydrazide	Amine	[42]
Polyacrylamide	Amide	Acid pH	Carboxylic acid	Amine	[42]
Polyester	Ester	Acid pH	Carboxylic acid + alcohol	Amine	[43]
Polyethylene	CH_2	Conc. Nitric acid	Carboxylic acid	Amine	[44]
Polystyrene		Conc. Nitric acid	Nitrated aromatic ring	Histidine, Tyrosine	[45, 46]
Nylon	Amide	Hydrazine	Hydrazide	Amine	[47]

with the enzyme once the enzymatic activity decays. The benefit of obtaining a leak proof binding between enzyme and matrix resulting from these reactions could be partially offset by the cost, in terms of generally low yield of immobilized activity and by the nonreversible character of this binding. Enzymes attached covalently by disulfide bonds to solid supports, represent one way to avoid this problem, as will be described in Chapter 7.

5.2 Entrapment and Cross-linking

The entrapment method is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass through but retains the enzyme [48]. This method differs from the coupling methods described above, in that the enzyme is not bound to the matrix or membrane. There are different approaches to entrapping enzymes such as gel [49] or fiber entrapping [50], and micro-encapsulation [51]. The practical use of these methods is restricted by mass transfer limitations through membranes or gels.

The more recently reported technique [52, 53] for immobilization of enzymes as cross-linked enzyme aggregates (CLEAs®) diverges slightly from the conventional immobilization methods. CLEAs are based on multipoint attachment through intermolecular cross-linking between enzyme molecules. Successful preparation of CLEAs from a broad range of enzymes, including penicillin acylases, lipases, laccases, and horseradish peroxidase is currently being evaluated by many researchers [54].

6 Methods of Reversible Immobilization

Because of the type of the enzyme-support binding, reversibly immobilized enzymes can be detached from the support under gentle conditions. The use of reversible methods for enzyme immobilization is highly attractive, mostly for economic reasons simply because when the enzymatic activity decays the support can be regenerated and re-loaded with fresh enzyme. Indeed, the cost of the support is often a primary factor in the overall cost of immobilized catalyst. The reversible immobilization of enzymes is particularly important for immobilizing labile enzymes and for applications in bioanalytical systems [31].

6.1 Adsorption (Noncovalent Interactions)

6.1.1 Nonspecific Adsorption

The simplest immobilization method is nonspecific adsorption which is mainly based on physical adsorption or ionic binding [55, 56]. In physical adsorption the enzymes are attached to the matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions, whereas in ionic bonding the enzymes are bound through salt linkages. The nature of the forces involved in noncovalent immobilization results in a process which can be reversed by changing the conditions that influence the strength of the interaction (pH, ionic strength, temperature, or polarity of the solvent). Immobilization by adsorption is a mild, easy to perform process, and usually preserves the catalytic activity of the enzyme. Such methods are therefore economically attractive, but may suffer from problems such as enzyme leakage from matrix when the interactions are relatively weak.

6.1.2 Ionic Binding

An obvious approach to the reversible immobilization of enzymes is to base the protein-ligand interactions on principles used in chromatography. For example, one of the first applications of chromatographic principles in the reversible immobilization of enzymes was the use of ion-exchangers [4, 57, 58]. The method is simple and reversible but, in general, it is difficult to find conditions under which the enzyme remains both strongly bound and fully active. More recently, the use of immobilized polymeric ionic ligands has allowed to modulate the interactions between protein and matrix and thus to optimize the properties of the derivative. A number of patents have been filed on the use of polyethyleneimine to bind a rich variety of enzymes and whole cells [59].

However, problems may arise from the use of a highly charged support when the substrates or products are themselves charged; the kinetics are distorted due to partition or diffusion phenomena. Therefore, enzyme properties such as its optimum pH or the pH stability range may change [60, 61]. Although this could be a problem it can also be useful to shift the optimal conditions of a certain enzyme towards more alkaline or acidic conditions, depending on the application [62].

6.1.3 Hydrophobic Adsorption

Another approach is the use of hydrophobic interactions. In this method, it is not the formation of chemical bonds but rather an entropically driven interaction that takes place. Hydrophobic adsorption has been used as a chromatographic principle for more than three decades. It relies on well-known experimental variables such as pH, salt concentration, and temperature [63]. The strength of interaction relies both on the hydrophobicity of the adsorbent and that of the protein. The hydrophobicity of the adsorbent can be regulated by the degree of substitution of the support and by the size of the hydrophobic ligand molecule. The successful reversible immobilization of β-amylase and amyloglucosidase to hexyl-agarose carriers has been reported [64, 65]. Several other examples of strong reversible binding to hydrophobic adsorbents have also been reported [66–68].

6.1.4 Affinity Binding

The principle of affinity between complementary biomolecules has been applied to enzyme immobilization. The remarkable selectivity of the interaction is a major benefit of the method. However, the procedure often requires the covalent binding of a costly affinity ligand (e.g., antibody or lectin) to the matrix [69].

6.2 Chelation or Metal Binding

Transition metal salts or hydroxides deposited on the surface of organic carriers become bound by coordination with nucleophilic groups on the matrix. Mainly titanium and zirconium salts have been used and the method is known as “metal link immobilization” [16, 70, 71]. The metal salt or hydroxide is precipitated onto the support (e.g., cellulose, chitin, alginic acid, silica-based carriers) by

heating or neutralization. Because of steric factors, it is impossible for the matrix to occupy all coordination positions of the metal, and therefore some of the positions remain free to coordinate with groups from the enzymes. The method is quite simple and the immobilized specific activities obtained with enzymes in this way have been relatively high (30–80 %) However, the operational stabilities achieved are highly variable and the results are not easily reproducible. The reason for this lack of reproducibility is probably related to the existence of nonuniform adsorption sites and to a significant metal ion leakage from the support. In order to improve the control of the formation of the adsorption sites, chelator ligands can be immobilized on the solid supports by means of stable covalent bonds. The metal ions are then bound by coordination, and the stable complexes formed can be used for the retention of proteins. Elution of the bound proteins can be easily achieved by competition with soluble ligands or by decreasing pH. The support is subsequently regenerated by washing with a strong chelator such as EDTA (ethylene diamino tetraacetic acid disodium salt) when desired. These metal chelated supports were named IMA (Immobilized Metal-Ion Affinity)-adsorbents and have been used extensively in protein chromatography [72, 73]. The approach of using different IMA-gels as supports for enzyme immobilization has been studied using *E. coli* β-galactosidase as a model [74].

6.3 Formation of Disulfide Bonds

These methods are unique because, even though a stable covalent bond is formed between matrix and enzyme, this bond can be broken by reaction with a suitable agent such as dithiothreitol (DTT) under mild conditions. Additionally, since the reactivity of the thiol groups can be modulated by changing the pH, the activity yield of the methods involving disulfide bond formation is usually high, provided that an appropriate thiol-reactive adsorbent with high specificity is used [75]. Immobilization methods based on this strategy are discussed in Chapter 7.

7 Properties of Immobilized Enzymes

The properties of immobilized enzymes are determined by the characteristics of carrier material as well as by the nature and number of interactions between the enzyme and the support. As a consequence of enzyme immobilization, the stability and kinetic properties of enzymes are usually changed, mostly due to the microenvironment and modifications imposed by the supporting matrix [11, 76].

This modification in the properties may be caused either by changes in the intrinsic activity of the immobilized enzyme or by the fact that the interaction between the immobilized enzyme and the substrate takes place in a micro-environment that is different from the bulk solution. So, one of the main problems associated

with the use of immobilized enzymes is the loss of catalytic activity, especially when the enzymes are acting on macromolecular substrates. Because of the limited access of the substrate to the active site of the enzyme, the activity may be reduced to accessible surface groups of the substrate only. This steric restriction may in turn, change the characteristic pattern of products derived from the macromolecular substrate [77]. There are several strategies to avoid these steric problems such as: the selection of supports composed by networks of isolated macromolecular chains, the careful choice of the enzyme residues involved in the immobilization, and the use of hydrophilic and inert spacer arms [78].

The observed changes in the catalytic properties upon immobilization may also be due to changes in the three-dimensional conformation of the protein provoked by the binding of the enzyme to the matrix. These effects have been demonstrated and, to a lesser extent exploited for a limited number of enzyme systems. Quite often, when an enzyme is immobilized, its operational stability at higher temperature and in the presence of organic solvents is highly improved [79]. The concept of stabilization has thus been an important driving force for immobilizing enzymes. True stabilization at the molecular level has been demonstrated, such as the case of proteins immobilized through multipoint covalent binding [80]. Studies carried out by several authors using different methods have demonstrated that there is a correlation between stabilization and the number of covalent bonds to the matrix [81–83].

8 Enzyme Immobilization Mimics Biology

Although the science of enzyme immobilization has developed as a consequence of its technical utility, one should recognize that the advantages of having enzymes attached to surfaces have been exploited by living cells as long as life existed. An inquiry into the biological role of enzyme immobilization may provide some lessons for the biotechnologists and serve as a second point of departure, in addition to the purely chemical one. In fact, there is experimental evidence that the immobilized state might be the most common one for enzymes in their natural environment. In an attempt to mimic biology, co-immobilization of a number of sequential or cooperating biocatalysts on the same support has been used as a strategy to improve stability and enhance reaction kinetics [84]. The attachment of enzymes to the appropriate surface ensures that they stay at the site where their activity is required. This immobilization enhances the concentration at the proper location, and it may also protect the enzyme from being destroyed. Numerous bi-enzyme systems have been reported; a remarkable example is the co-immobilization of peroxidase and glucose oxidase onto carbon nanotubes to be used as a glucose biosensor [85, 86].

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UNIT I- FERMENTATION –ZYMOLOGY

(SCIENCE THAT DEALS WITH FERMENTATION)

FERMENTATION TECHNOLOGY

(SCIENCE THAT DEALS WITH FERMENTATION)

is a field which involves the use of microorganisms and enzymes for production of compounds which have application in the energy, material, pharmaceutical, chemical and the food industry

What is Fermentation?

The word Fermentation is derived from Latin word *fervere which means to boil.*

Traditionally Fermentation involves:

Conversion of Complex organic substrates into an economically useful metabolites using enzymes of yeast, bacteria, or microorganisms at anaerobic conditions.

(OR)

In Modern terms:

Fermentation applies to the- growth of microorganisms (not plant cell/animal cell) in liquid media under either conditions (aerobic & anaerobic) to produce economically useful products.

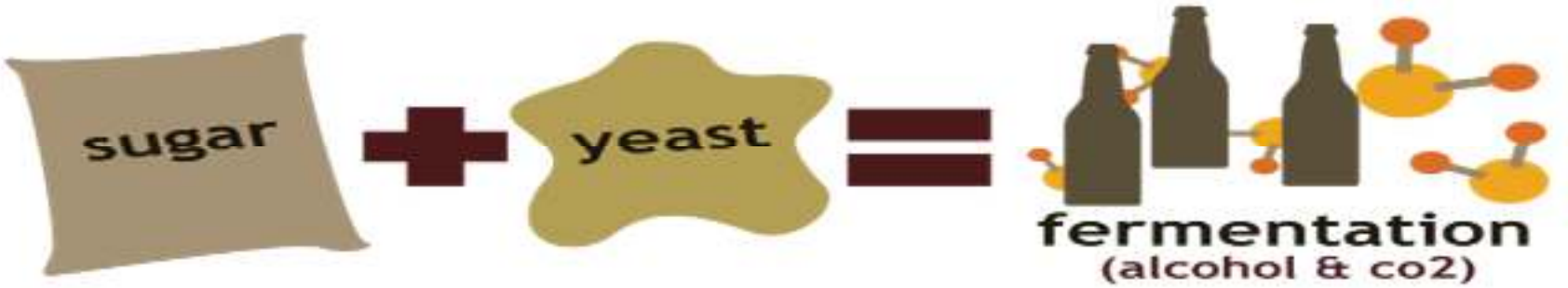
(OR)

In Biochemical sense:

It is the process by which microorganisms generate energy from organic compounds.

Overall,

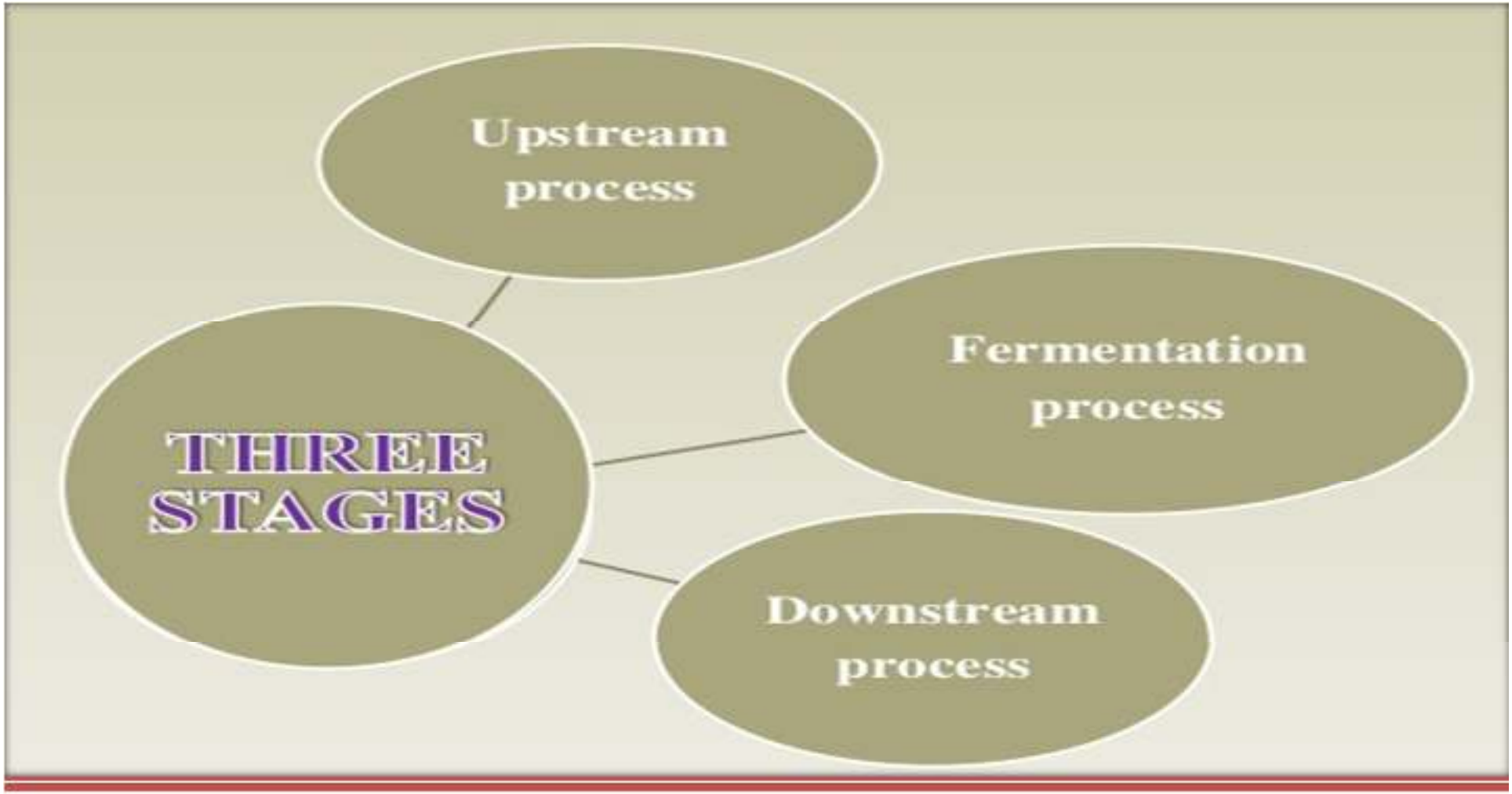
Fermentation is: Cultivation of microbes which serves as source of industrially /therapeutically useful products/metabolites.



-End Products Range is

Microbial cells / biomass-:	Yeast cells, single cell proteins(SCP)
Microbial metabolites-:	Glutamic acid, lysine, phenyl alanine, proteins, Vitamins
r DNA products-:	Hormones (Growth hormone, insulin),
	Therapeutic protein (Clotting factors, Inter ferons)
Transformation products- :	Steroidal drugs, Prostaglandins
Microbial enzymes-:	Bacterial(amylase, protease, lipase, isomerase)
	Fungal (amylase, protease, pectinase, amyloglycosidase)
	Animal (Streptokinase)

The end products formed as a result of their metabolism during their life span are released into the media, which are extracted for use of human being and that have high commercial value.


Upstream process
THREE STAGES
Fermentation process
Downstream process

Operational stages in Fermentation/Fermentation Process/Components :

I. UPSTREAM PROCESS-Includes all the operations before fermentation

A. Inoculum development (microbes)

✓Sources of Industrially useful microbes

(Culture collection, Isolation, Screening and Strain improvement)

✓ Preservation

B. Culture Media – Preparation and Preservation

II. FERMENTATION: FERMENTER – Types,

III. DOWN STRAEM PROCESS-

Which includes all the operations after the fermentation has occurred-

✓ Product recovery and Purification

✓ Effluent treatment & waste disposal

I. UPSTREAM PROCESS

The Pre-Fermentation Stage-Inoculum development & Culture medium preparation

I. **A. Characteristics of microbes/strains**

B. Sources of microbes/strains

C. Isolation of microbes/strains

D. Screening method-

Two methods:-

Primary screening: checking the quality of microbes done in agar plate

Secondary screening: checking the quntative of microbes done in liquid media

E. Improvement of microbes/strains

F. Preservation of microbes/strains

G. Inoculum development

II. Preparation of culture media/ingredients

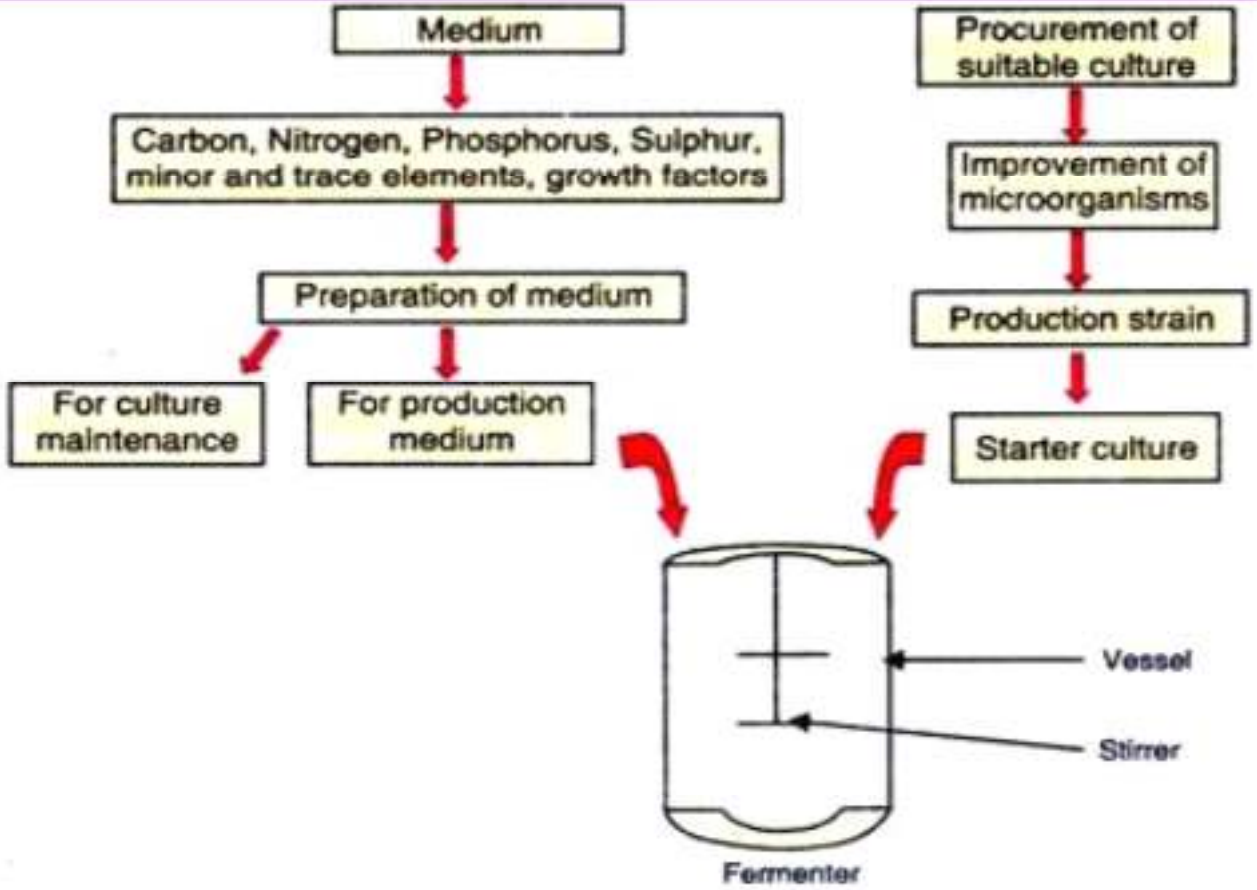


Fig. 20.4: Steps of up-stream process (USP).

A. Characteristics of Strain/ Microbes

The microbe (more specific strain) that is to be employed for fermentation should possess the following industrially useful characteristics:-

- ✓ **Should be genetic stability**
- ✓ **Consistency in production**
- ✓ **High yielding capacity for the desired product**
- ✓ **Permit easy recovery of the formed product**
- ✓ **Resistance to infections**
- ✓ **Non foaming**
- ✓ **Tolerate to low oxygen**
- ✓ **Have an optimum temperature for growth & operation above 400c, so that cooling costs are reduced.**

B. Sources of Microbes/Strains/Culture

The microbes isolated from environment (soil), however the industrial strains may also "isolate" microorganisms from culture collections:

- ✓ **National collection of type culture (NCTC)-UK**
- ✓ **National collection of industrial and marine bacteria (NCIB, NCMB)-UK**
- ✓ **Collection of international mycological institute (IMI) –UK**
- ✓ **American type culture collection (ATCC)- USA**
- ✓ **Japan collection of microorganisms (JCM)-Japan**
- ✓ **The Microbial Type Culture Collection and Gene Bank (MTCC)- Chandigarh, India**
- ✓ **Anaerobic Bacterial Resource Centre, University of Hyderabad, TELAGANA, India**
- ✓ **Culture Collection, Department of Microbiology, Bose Institute, WB, India**
- ✓ **Indian Type Culture Collection, Division of Mycology and Plant Pathology, New Delhi, India**
- ✓ **Microbial Culture Collection, National Centre for Cell Science, Pune University Campus, India**

C. Isolation of microbes/strains

There are over a million species of microorganisms widely distributed in nature. Less than 1% of the world's microorganisms have been studied. In fact, only a few hundred species are important for industrial use.

Microorganism	Product
Algae-Spirulina maxima	SCP single cell proteins
Bacteria- Acetobacter aceti	Acetic acid
Actinomycetes- Strptomyces aurofaciens Strptomyces griseus	 Tetracycline streptomycin
Fungi- Aspergillus niger Aspergillus oryzae Candida lipolytica	Citric acid Amylase, cellulase Lipase

The good sources for the isolation of microorganisms are soils, lakes and river muds.

It is estimated that a gram of soil contains 10^6—10^8bacteria, 10^4—10^6 actinomycete spores and 10^2—10^4 fungal spores.

The common techniques employed for the isolation of microorganisms are given below:

1. Direct sponge of the soil
2. Soil dilution
3. Gradient plate method (Pour plate and streak plate technique)
4. Aerosol dilution
5. Flotation
6. Centrifugation
7. Enrichment Methods

The general scheme adopted for isolating microorganisms from soil or water source is given below:

1. The sample (soil or water) is diluted with sterile water to which an emulsifying agent (Tween) is added.

2. Sample is thoroughly mixed and allowed to stand at room temperature.

3. Supernatant is diluted, 10^{-1} to 10^{-10}

4. Various culture media are inoculated with diluted samples and incubated.

5. Colonies from the plates are isolated and identified.

6. The required pure strains are maintained and preserved.

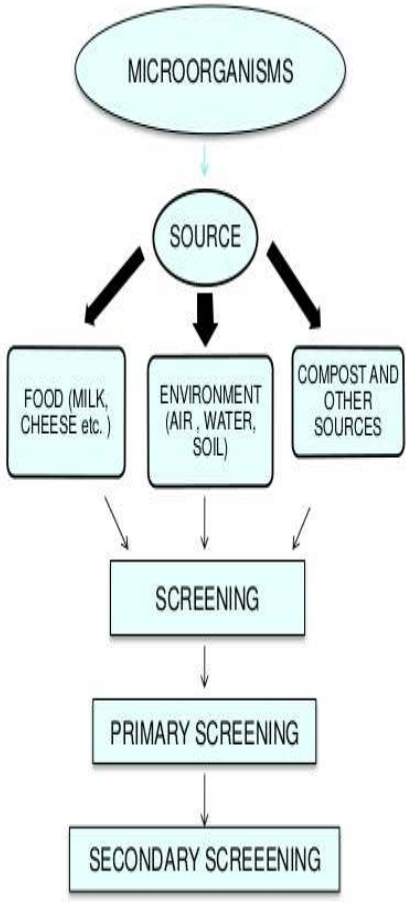
D. Screening of Strains/Microorganisms:-

Detection and isolation of a our interest microorganism from a natural environment like soil containing large number of microbial population is called as screening. It is very time consuming and expensive process. For example, Eli Lilly & Co. Ltd discovered three species of antibiotic producing organisms in a span of 10 years and after screening 4,00,000 organisms.

Although there are many screening techniques, all of them are generally grouped into two broad categories.

They are:

1. Primary screening,
2. Secondary screening.



1. Primary screening (PS)

↓

1.“Primary screening allows the detection & isolation of microorganisms that possess potentially interesting industrial application”

2. Primary screening separate out only a few microorganisms having real commercial value.

3. Primary screening determines which microorganisms are able to produce a compound without providing much idea of the production or yield potential of the organisms.

2. Secondary screening (SS)

↓

1. “Secondary screening allows further sorting out of microorganisms obtained from PS having real value for industrial processes and discarding of those lacking this potential”.

2. SS is conducted on agar plates, in flasks or small fermenter containing liquid media

3. SS can be qualitative or quantitative in its approach

4. Secondary screening should give information about the evaluation of the true potential of the microorganisms for industrial usage.

5. SS helps in predicting the approaches to be utilized in conducting further research on the microorganisms and its fermentation processes.

Primary Screening Techniques

1. The crowded plate technique
2. Indicator dye technique
3. Enrichment culture technique
4. Auxanographic technique
5. Technique of supplementing volatile and organic substrates

Secondary Screening Techniques

Secondary screening gives very useful information pertaining to the newly isolated microorganisms that can be employed in fermentation processes of commercial value. These screening tests are conducted by using petri dish containing solid media or by using flasks or small fermenters containing liquid media. Each method has some advantages and disadvantages.

Liquid media method is more sensitive than agar plate method because it provides more useful information about the nutritional, physical and production responses of an organism to actual fermentation production conditions.

- ✓**Giant Colony Technique**
- ✓**Filtration Method**
- ✓**Liquid Medium Method**

E. Culture/Strain/Microbe Improvement:-

The Science and Technology of manipulating and improving microbial strains in order to enhance their metabolic capacities is known as Strain Improvement.

❖*Purpose of Strain Improvement:*

- ✓ Increase the productivities
- ✓ Regulating the activity of the enzymes
- ✓ Introducing new genetic properties into the organism by Recombinant DNA technology / Genetic engineering.

❖ *The improved strain possess the following characteristics:*

1. Shorter time of fermentation
2. Capable of metabolizing low cost substrates.
3. Reduced oxygen demand
4. Decreased foam formation
5. Non-production of undesirable compounds.
6. Tolerance to high concentration of carbon and nitrogen sources.
7. Resistant to infection of bacteriophages.

❖ ***Strain improvement can be performed in following ways/ Approaches:***

1. **Mutation and mutant selection**
2. **Protoplast fusion**
3. **Recombinant DNA technique**

1. MUTATION

A MUTATION is a Sudden and Heritable change in the traits of an organism.

✓ Application of Mutagens to Induce mutation is called MUTAGENESIS.

✓ Agents capable of inducing mutations are called MUTGENS

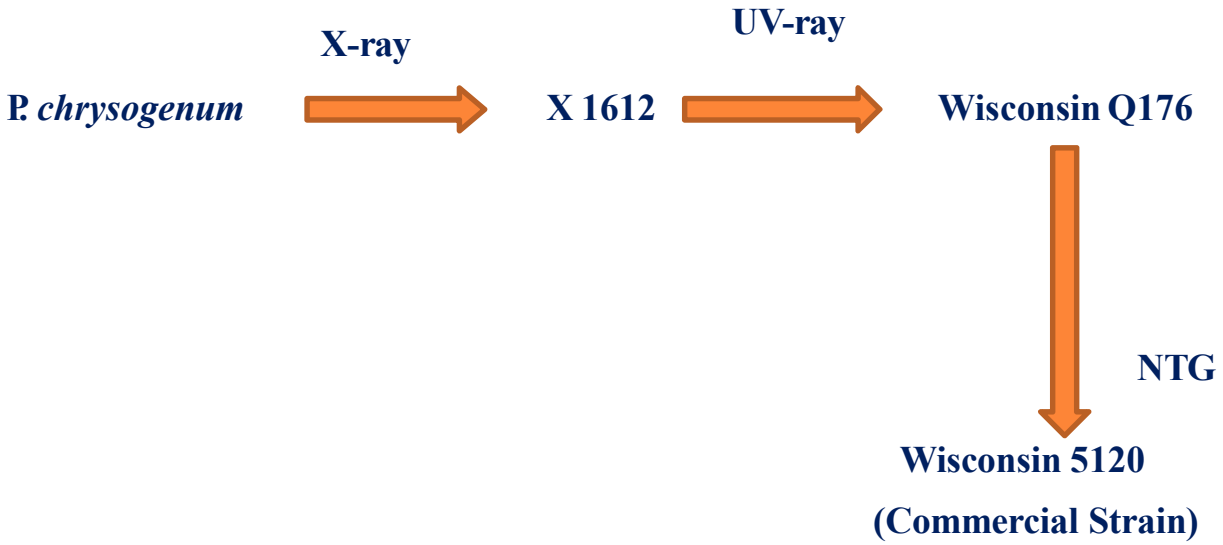
✓ Chemical mutagens–Alkylating agents, Acridine Dyes, etc.

✓ Mutation occurring without any specific treatment are called " Spontaneous Mutation."

✓ Mutation are resulting due to a treatment with certain agents are known as "Induced Mutation."

- **Mutagenesis is a suitable method to develop new strain with superior qualities from a wild type organism.**
- **Usually X-ray, UV-ray and chemical mutagens are used for inducing variation.**
- **By this method, if wild type is unstable it would made into stable.**
- ***P. chrysogenum* strain was screened from wild type culture by culturing in a plates under biochemical test.**
- **It produces about 80-100 units of penicillin per ml.**

- ***Another example is the production of Wisconsin 5120.***



- **N-methyl-N-nitro-N-nitroguanidine (NTG) is a commercial strain used in industries for producing penicillin.**
- **It produces 2,500-5,000 units of penicillin per ml.**

2. PROTOPLAST FUSION

1. Protoplast are the cells devoid of cell wall.
2. It is produced using lysosome (cell wall degrading enzyme) in isotonic solution.
3. Fusion of protoplast obtained from somatic cells of fungi and bacteria is very useful to develop industrial strain in many cases.

Hamlin and **ball fused the protoplasts of two strains of**

Cephalosporium acremonium using PEG (polyethylene glycol).

The resultant protoplast produced more cephalosporin.

Chang *et.al* *fused the protoplast of two strains of Penicillium chrysogenum.*

The resultant protoplast produced penicillin-v.

3. RECOMBINANT DNA TECHNIQUE

✓ Many industrial strains have been developed using r DNA technology .

✓ The gene is transferred into the harmless microbe through DNA vector is called **cloning vehicle.**

✓ So organism become improved strain for the production of particular product.

Eg: the **human insulin gene was transferred into the *E.coli*** K12 strain using cloning vehicle **pBR322 .** Hence ***E.coli K12*** started to producing insulin.

F. Preservation of Microbes/Strains/Culture

Microbes are required for the production of fermentation products. They are very valuable for specific product. Not all the microbes will give one product produced efficiently by specific microbe. The isolation of a desired organism for a fermentation process may be time consuming and very expensive procedure and it is therefore essential that it retain the desirable characteristics that led to its selection.

In addition, the culture used for the fermentation process should remain viable and free from contamination. Thus, industrial cultures must be preserved and maintained in such way as to eliminate genetic change, protect against contamination, and retain viability.

Different techniques are used for maintenance and preservation of different organisms based on their properties. Selected method should also conserve the properties of the organisms.

Techniques for the Preservation of microbes broadly divided into two:

1. **Methods where organisms are in Continuous metabolic active state**

I. **Continuous metabolic active state preservation technique**
II. **Periodic transfer to fresh media**
III. **Overlaying culture with mineral oil**
IV. **Storage in sterile soil**
V. **Saline suspension**

In this technique, organisms preserved on nutrient medium by repeated sub-culturing. In this technique, any organisms are stored by using general nutrient medium. Here repeated sub culturing is required due to depletion or drying of nutrient medium.

2. **Methods where organisms are in Suspended metabolic state**

I. **Drying in vacuum**
II. **Lyophilization**
III. **Use of Liquid nitrogen**
IV. **Storage in silica gel**

Organisms preserved in suspended metabolic state by either drying or storing at low temperature. Microbes are dried or kept at low temperature carefully so that their revival is possible.

1. Methods where organisms are in Continuous metabolic active state

I. Periodic transfer to fresh media : Organisms grown in general media on slant, incubated for particular period at particular temperature depending on the characteristics of the selected organisms, then it is stored in refrigerator. These cultures can be stored for certain interval of time depending on the organism and its growth conditions. After that time interval, again these organisms transferred to new fresh medium and stored in refrigerator.

II. Overlaying culture with mineral oil : Organisms are grown on agar slant then they are covered with sterile mineral oil to a depth of 1 cm. above the tip of the surface. This method is simple; one can remove some organisms in aseptic condition with the help of sterile wire loop and still preserving the initial culture. Some species preserved satisfactorily for 15 – 20 years by this method.

III. Storage in sterile soil: This method is widely used for preserving spore forming bacteria and fungi. In this method, organisms will remain in dormant stage in sterile soil. Soil sterilized then spore suspension added to it aseptically, this mixture dried at room temperature and stored in refrigerator. Viability of organisms found around 70 – 80 years.

IV. Saline suspension : Normal Saline used to provide proper osmotic pressure to organism's otherwise high salt concentration is inhibitory for organisms. Organisms kept in screw cap bottles in normal saline, stored at room temperature, wherever required transfer made on agar slats, and incubated.

2. Methods where organisms are in Suspended metabolic state

I. Drying in vacuum : In this technique, organisms dried over chemical instead of air dry Cells passed over CaCl2 in a vacuum and then stored in refrigerator. Organisms survive for longer period.

II. Lyophilization : Lyophilization is vacuum sublimation technique. Cells grown in nutritive media and then this culture distributed in small vials. These vials culture then immersed in a mixture of dry ice and alcohol at -78oC. These vials immediately connected to a high-vacuum line, and when they are completely dried, each vial sealed under vacuum. This is most effective and widely used technique due to long time survival, less opportunity for changes in characteristics of organisms and small storage area. Organisms can survive for period of 20 years or more.

III. Use of Liquid nitrogen : Microorganisms grown in nutritive media and then this culture frozen with Cryoprotective agents like Glycerol and Dimethyl Sulfoxide. Frozen culture kept in liquid Nitrogen refrigerator. Organisms can remain alive for longer period.

IV. Storage in silica gel : Both bacteria and yeast stored by this method. By this technique, organisms can survive for 1 – 2 years. Finely Powdered Heat sterilized Silica powder mixed with thick suspension of cell at low temperature.

Quality control of the preserved stock culture

Whichever technique used for the preservation and maintenance of industrially important organisms it is essential to check the quality of the preserved organisms stocks. Each batch of newly preserved cultures routinely checked to ensure their quality. A single colony transferred into a shake-flask to ensure growth of particular kind of microorganism; further shake-flask subculture used for the preparation of huge quantity of vials. For the assessment of purity, viability, and productivity of cultures, few vials are tested. If samples fail any one of these tests, the entire batch destroyed. Thus, by the use of such a quality-control system stock cultures retain and used with confidence.

G. Inoculum development

Definition of Inoculum :-

Inoculum is the mixture of cultured microbes Along with media in which it is growing.
(OR) Development of active logarithmic microbial culture that is suitable for the final industrial production level is known as Inoculum development.

The aim of Inoculum preparation is to select industrial microorganisms with high productivity and to minimize low productive, mutant strains for a particular fermentation process.

Since profit making and cost reduction are two critical issues in any industrial microbiology process, it is important to use industrial microbes with higher biomass production and higher product yield so that the production process can be optimized appropriately with minimal or no loss. Several steps are usually involved in Inoculum preparation.

II. Preparation of Culture media & Ingredients

Detailed investigation is needed to establish the most suitable medium for an industrial fermentation process, but certain basic requirements must be met by any such medium.

All microorganism requires water, source of energy, C, N, Mineral, Vitamins, Oxygen (if aerobic).

On a small scale its easy to maintain but on large scale very difficult to maintain the satisfactory growth.

On a large scale one must normally use sources of nutrients to create a medium which will meet as many as possible of the following criteria:

1. It will produce the maximum yield of product or biomass per gram of substrate used.
2. It will produce the maximum concentration of product or biomass.
3. It will permit the maximum rate of product formation.
4. There will be the minimum yield of undesired products.
5. It will be of a consistent quality and be readily available throughout the year.
6. It will cause minimal problems during media making and sterilization.
7. It will cause minimal problems in other aspects of the production process particularly aeration and agitation, extraction, purification and waste treatment.

✓The use of cane molasses, beet molasses, cereal grains, starch, glucose, sucrose & lactose as carbon source & NH_4 salts, nitrates, corn steep liquor, soya bean meal, slaughter house waste and fermentation residues as N source – meet the above criteria for production media b/c they are cheap material.

✓Use simpler procedures, select suitable sporulation and inoculation media.

✓Medium selected will affect the design of fermenter.

✓Microbial oxidation of Hydrocarbons is a highly aerobic and exothermic process thus production fermentar should have a vey high O_2 transfer capacity with excellent cooling facilities.

Culture Medium: **Nutrients prepared for microbial growth**

Inoculum: **Suspension of microorganisms**

Inoculation: **Culture Media Introduction of microbes into culture medium**

Culture: **Microbes growing in/on culture medium**

A pure culture contains only one species or strain

Mixed culture contains several species

Contaminated culture contains unwanted species of organisms

I. According to composition:

Chemically Defined Media (synthetic): Exact chemical composition is known e.g. glucose inorganic salt phosphate for E. coli

Complex Media (non-synthetic): chemical composition is not specifically defined; Extracts and digests of yeasts, meat, or plants e.g. Nutrient broth, Nutrient agar, McConkey, EMB

II. According to Consistency:

solid- with 1.5 to 3.0% agar

e.g. NA (Nutrient Agar)

liquid- no solidifying agent

e.g. NB (Nutrient Broth)

semi solid- with less than 1.5% agar

e.g. SIM (Sulfide Indole Motility Medium)

- ✓Lab medium may not be ideal in a large fermenter with a low gas transfer pattern.
- ✓A medium with high viscosity will also need a higher power input for effective stirring.
- ✓pH variation, foam formation, oxidation, reduction potential & the morphological form of the organism, metabolic inhibitors – these are also very important.
- ✓Medium will also affects product recovery & effluent treatment.
- ✓Historically – undefined complex natural materials used – although they are cheaper but unpredictable biomass/yield problems.
- ✓Product recovery very difficult, residual components my interfere with the recovery and contribute to the BOD of the effluent.
- ✓Although defined media is more expensive but it give more predictable yield than undefined media.
- ✓Control of pH and foam during growth in a fermenter were indentified as two important parameters.
- ✓Molasses would normally be used as the cheapest carbohydrate to grow yeast biomass in a large scale process. But this is not acceptable for Recombinant protein production b/c of difficulties and cost for purification.
- ✓Defined medium with glucose, sucrose, animal salts, trace elements, pure vitamins & NH_4 as N source & for pH control, also used antifoaming agent.

Culture Media Formulation

Medium formulation is an essential stage in the design of successful laboratory experiments, pilot – scale development and manufacturing process.

The constituent of the medium must satisfy the elemental requirements for cell biomass & metabolite production and there must be an adequate supply of energy for biosynthesis.

$$C + N + O_2 + \text{other req} \text{ ------- } \text{biomass} + \text{product} + CO_2 + H_2O + \text{Heat}$$

This equation should be expressed in quantitative terms, which is important in the economical design of media if component wastage is to be minimal.

Thus, to calculate the minimal quantities of nutrients which will be needed to produce a specific amount of biomass, it should calculate substrate concentration necessary to produce required product yields.

Oxygen :– Provided by aerating the culture, the design of medium will influence the oxygen demand of a culture in that the more reduced C sources will result in a higher O_2 demand.

Water: -ALL fermentation process, except SSF, require vast quantities of water.

required in:

Heating , cooling, cleaning & rinsing

It required in large quantities, pH, Dissolved salts and effluent contamination

Mineral requirement of water is very important in brewing & most critical in the mashing process.

Nowadays, water may be treated by deionization, salt added or pH adjusted, depend upon the type of breweries. Reuse or efficient use of water is normally of high priority.

SCP produced 60,000 tones by ICI, used huge amount of water on single use bases,

(30g biomass (dw) dm^{-3} would require 2700 x 10^6 dm^3 of water per annum.

Eg. *Methylophilus methlotrophus* could grown successfully with 86% continuous **recycling** of supernatant with addition to make up depleted nutrients. It result in reduce capital cost and operating cost by 50%.

Energy Sources:

Energy comes either from oxidation of medium components or from light.

Most industrial microorganism are chemo-organotrophs, therefore the commonest source of energy will be the carbon (CHO), lipids & protein.

Some micro-organisms can also use hydrocarbons or methanol as carbon & energy sources.

Carbon Source:

Factor influencing the choice of carbon source:

Rate at which the C source is metabolized can often influence the formation of biomass or production of primary or secondary metabolites.

Fast growth due to high concentrations of rapidly metabolized sugars is often associated with low productivity of secondary metabolites.

Main product of fermentation process depend on the choice of *C* source.

Eg. Ethanol, or SCP - 60-77% of the production cost depend on cost of C source.

The purity of the C source may also affect the choice of substance.

The method of media preparation, particularly sterilization may affect the suitability of CHO for individual fermentation process.

Its often best to sterilize sugar separately b/c they may react with NH_4 ions & Amino acids which will inhibit the growth of many microorganism.

Starch when sterilize it become gelatinizes give rise to very viscous liquid.

The choice of substrate may also be influenced by Govt rules, Eg: Beet sugar and molasses is encouraged. Scotch malt whisky may be made only from barley malt, water and yeast.

Carbohydrates:

Its common practice to use CHO in microbial fermentation, example: starch from maize grains

It may also obtained from, others cereals, potatoes & cassava.

Hydrolyzed cassava starch is used as a major C source for glutamic acid production.

Syrups produced by acid hydrolysis may also contain toxic products which may make them unsuitable for particular processes.

Sucrose is obtained from sugar cane & sugar beet. It is commonly used in fermentation media in a very impure form as beet or cane molasses which are the residues left after crystallization of sugar solution in sugar refining.

Molasses is used in the production of high volume/low value products such as ethanol, SCP, organic and AA and some microbial gums.

TABLE 4.5. *Carbohydrate composition of barley malt* (Harris, 1962) *(expressed as % dry weight of total)*

Starch	58–60
Sucrose	3–5
Reducing sugars	3–4
Other sugars	2
Hemicellulose	6–8
Cellulose	5

TABLE 4.6. *Analysis of beet and cane molasses* (Rhodes and Fletcher, 1966) *(expressed as % of total w / v)*

	Beet	Cane
Sucrose	48.5	33.4
Raffinose	1.0	0
Invert sugar	1.0	21.2

Molasses or sucrose also may be used for production of higher value/low bulk products such as antibiotics, specially enzymes, vaccines & fine chemicals.

However, molasses based fermentation will often need a more expensive & complicated extraction / purification procedure to remove impurities & effluents which make the procedure costly.

Corn steep liquor is a by product after starch extraction from maize. Although primarily used as a N source, it does contain lactic acid, small amounts of reducing sugars & complex polysaccharides.

Oils and Fats:

Oils were first used as carriers for antifoams in antibiotics processes.

Vegetables oils may also be used as C substrates, particularly for their content of the fatty acid, oleic, linoleci and linolenic acid b/c of cost are competitive with those of CHO.

Bader 1984 – factors favouring the use of oils instead of CHO. A typical oil contains approximately 2.4 times the energy of glucose on a per weight basis.

Oils also have a volume advantage as it would take 1.24 dm^3 of soya bean oil to add 10KCal energy to a fermenter whereas it would take 5dm^3 of glucose or sucrose assuming that they are being added 50%w/w solutions.

Oil also has antifoam properties which may make downstream processing simpler.

Glycerol trioleat is known to be used in some fermentation where substrate purity is an important consideration.

Methly oleate has been used as the sole C substrate in cephalosporin production

Nitrogen sources:

Most industrially used microorganism can utilize inorganic or organic sources of nitrogen.

Inorganic N may be supplied as NH_3 gas, NH_4 salts or nitrates. NH_3 has been used for pH control & as the major N source in a defined medium for the commercial production of human serum albumin by yeast.

NH_4 salts i.e. $(NH_4)_2SO_4$ will produce acid conditions & NH_4NO_3 normally cause an alkaline drift.

When the NH_4 ion has been exhausted, there is an alkaline drift as the nitrate is used as n alternative N source.

Organic N may be supplied as amino acid, protein or urea. Other proteinaceous N compounds serving as sources of amino acid include corn-steep liquor, soya meal, peanut meal, cotton seed.

TABLE 4.9. *Best nitrogen sources for some secondary metabolites*

Product	Main nitrogen source(s)	Reference
Penicillin	Corn-steep liquor	Moyer and Coghill (1946)
Bacitracin	Peanut granules	Inskeep *et al.* (1951)
Riboflavin	Pancreatic digest of gelatine	Malzahn *et al.* (1959)
Novobiocin	Distillers' solubles	Hoeksema and Smith (1961)
Rifomycin	Pharmamedia Soybean meal, $(NH_4)_2SO_4$	Sensi and Thiemann (1967)
Gibberellins	Ammonium salt and natural plant nitrogen source	Jefferys (1970)
Butirosin	Dried beef blood or haemoglobin with $(NH_4)_2SO_4$	Claridge *et al.* (1974)
Polyenes	Soybean meal	Martin and MacDaniel (1977)

Minerals:

All micro-organism requires certain mineral elements for growth & metabolism.

In many media, Mg, P, K, S, Ca & Cl are essential components, Co, Cu, Zn, Fe, Mn, Mb & Zn are also essential P is used as a Buffer to minimize pH changes when external pH is not being used.

TABLE 4.10. *The range of typical concentrations of mineral components* (g dm^{-3})

Component	Range
*KH_2PO_4	1.0–4.0 (part may be as buffer)
$MgSO_4 \cdot 7H_2O$	0.25–3.0
KCl	0.5–12.0
$CaCO_3$	5.0–17.0
$FeSO_4 \cdot 4H_2O$	0.01–0.1
$ZnSO_4 \cdot 8H_2O$	0.1–1.0
$MnSO_4 \cdot H_2O$	0.01–0.1
$CuSO_4 \cdot 5H_2O$	0.003–0.01
$Na_2MoO_4 \cdot 2H_2O$	0.01–0.1

*Complex media derived from plant and animal materials normally contain a considerable concentration of inorganic phosphate.

- In specific processes the concentration of certain minerals may be very critical.
- Garner 1950 – Ca salts is used to precipitate the excess inorganic PO_4 so indirectly improve the yield of Streptomycin.
- It also influence the production of citric acid, ergot, monomycin, novobiocin, oxytetracycline, polyene, ristomycin, rifamycin, streptomycin, vacomycin & viomycin.

Liras 1990 – enzymes were repressed by PO_4, a PO_4 control sequence has also been isolated & characterized from the PO_4 regulated promoter that control biosynthesis of candicidin.

Wienberg 1970 – nine trace element proved to be critical, concentration of Mn & Zn are the most critical in secondary metabolism.

Cl does not appear to play a nutritional rule in the metabolism of fungi but however required by some of the halophilic bacteria. Eg. Chlortetracycline & griseofulvin.

Other Cl containing metabolites are caldriomycin, nornidulin & mollisin.

TABLE 4.11. *Trace elements influencing primary and secondary metabolism*

Product	Trace element(s)	Reference
Bacitracin	Mn	Weinberg and Tonnis (1966)
Protease	Mn	Mizusawa *et al.* (1966)
Gentamicin	Co	Tilley *et al.* (1975)
Riboflavin	Fe, Co	Hickey (1945)
	Fe	Tanner *et al.* (1945)
Mitomycin	Fe	Weinberg (1970)
Monensin	Fe	Weinberg (1970)
Actinomycin	Fe, Zn	Katz *et al.* (1958)
Candicidin	Fe, Zn	Weinberg (1970)
Chloramphenicol	Fe, Zn	Gallicchio and Gottlieb (1958)
Neomycin	Fe, Zn	Majumdur and Majumdar (1965)
Patulin	Fe, Zn	Brack (1947)
Streptomycin	Fe, Zn	Weinberg (1970)
Citric acid	Fe, Zn, Cu	Shu and Johnson (1948)
Penicillin	Fe, Zn, Cu	Foster *et al.* (1943)
		Koffler *et al.* (1947)
Griseofulvin	Zn	Grove (1967)

Chelators

Many media can't be prepared or autoclaved without the formation of a visible precipitate of insoluble metal PO_4 .

The problem of insoluble metal PO_4 may be eliminated by incorporating low conc of chelating agents i.e. EDTA, citric acid, polyphosphate etc

These chelating agents form complexes with the metal ions in a medium. The metal ions then my be gradually utilized by the micro-organism.

Chelating agent does not cause inhibition of growth.

Growth Factors

Vitamins, specific AA, fatty acids or sterols.

Many of the natural C & N sources used in media formulation contain all or some of the required growth factors.

If only one vitamins is required it may be move economical to add a pure vitamin instead of large bulk of cheaper multiple vitamins source. E.g Ca pantothenate has been used in one medium formulation for vinegar production.

In glutamic acid, limited conc of biotin must be present in the medium, some requires thiamin.

Buffers

The control of pH may be extremely important if optimal productivity is to be achieved.

Many media are buffered at about pH 7.0 by the incorporation of $CaCO_3$, if pH decreases the CO_3 is decomposed.

PO_4 which are the part of many media also play an important role in buffering. High PO_4 conc are critical in the production of many secondary metabolites.

C & N sources will also a basis for pH control as buffering capacity can be provided by the protein, peptides & AA such as in corn steep liquor.

The pH may also be controlled externally by addition of NH_3 or NaOH & H_2SO_4.

The addition of precursors & metabolic regulators to media

Some components of a fermentation medium help to regulate the production of the product rather than support the growth of the micro-organism. Such additive include, precursors, inhibitors,

Precursors

Some chemicals when added to certain fermentation are directly incorporated into the desired product.

Precursor	Product	Microorganism
Phenyl acetic acid	Penicillin G	*Penicillium chrysogenum*
Cyanides	Vitamin B12	*Propiano bacterium*
Beta ionone	Carotenoids	*Phycomyces blakesleeanus*
L-Threonine	Cyclosporine C	*Tolypocladium inflatum*

Inhibitors

When certain inhibitors are added to fermentations more of specific product may be produced.

Glycerol production depends on modifying the ethanol fermentation by removing acetaldehyde.

Inhibitors have also been used to affect cell wall structure and increase the permeability for release of metabolites.

The best example is the use of penicillin & surfactants in glutamic acid production.

Product	Inhibitor	Main effect	Microorganism
Glycerol	Sodium bisulphide	Acetaldehyde production repressed	*Saccharomyces cerivisiae*
Tetracycline	Bromide	Chlortetracycline formation repressed	*Streptomyces aurofaciens*
Glutamic acid	Penicillin	Cell wall permeability	*Micrococcus glutamicus*

Inducers

The majority of enzymes which are industrial interest are inducible.

Induced enzymes are synthesized only in response to the presence in the environment of an inducer.

Inducers are often substrate such as starch or dextrins for amylase, maltose for pullulanase & pectin for pectinase.

Most inducers which are included in microbial enzyme media are substrate or substrate analogues but intermediates and products may sometime be used as inducers.

E.g. Maltodextrins will inudce amylase & fatty acids induce lipase.

It is not possible to produce a no of heterologous protein (HLP) in yeasts, fungi & bacteria. But HLP may show some degree of toxicity to the host, it may restrict the growth and biomass.

Enzyme	Inducer	Microorganism
Alpha amylase	Starch	*Aspergillus spe.*
Proteases	Maltose	*Acetobacter aerogens*
Cellulase	Cellulose	*Trichoderma viride*

Oxygen requirement

Very important in controlling growth rate & metabolic production. Medium may influence:

Fast metabolism: culture may become oxygen limited b/c sufficient oxygen can't be made available in the fermeter if certain substrate such as rapidly metabolized sugars which lead to a high oxygen demand are available in high conc.

Rheology: the individual components of the medium can influence the viscosity of the final medium & its subsequent behavior with respect to aeration & agitation.

Rheology:

There can be considerable variation in the viscosity of compounds that may be included in fermentation media, eg. Polymer in solution i.e. polysaccharides

As polysaccharides degraded, the effect on rhelogical properties will change.

Antifoam:

In most microbiological process, foaming is a problem. It may be due to a component in the medium or some factor produced by the microorganism. E.g. most common due to protein i.e corn steep liquor, pharmamedia, peanut, soybean, yeast extract or meat extract, Paraffinic acid, amide waxes, sulfonated oils, organic phosphate, silicone oils, and mineral oils.

The foaming can cause remove of cells from the medium which will lead to autolysis & the further release of microbial cell proteins will probably increase the stability of the foam.

If uncontrolled, then numerous changes may occur & physical & biological problems may be created.

These include reduction in the working volume of the fermenter due to exhausted gas bubbles circulating in the system, lower mass & heat transfer rates, invalid process data due to interference at sensing electrode & incorrect monitoring & control.

Biological problem included, position of cells in upper parts of the feremnter, problems of sterile operation with the air filter exist of the fermenter become wet, so danger of microbial infections, so loss of product.

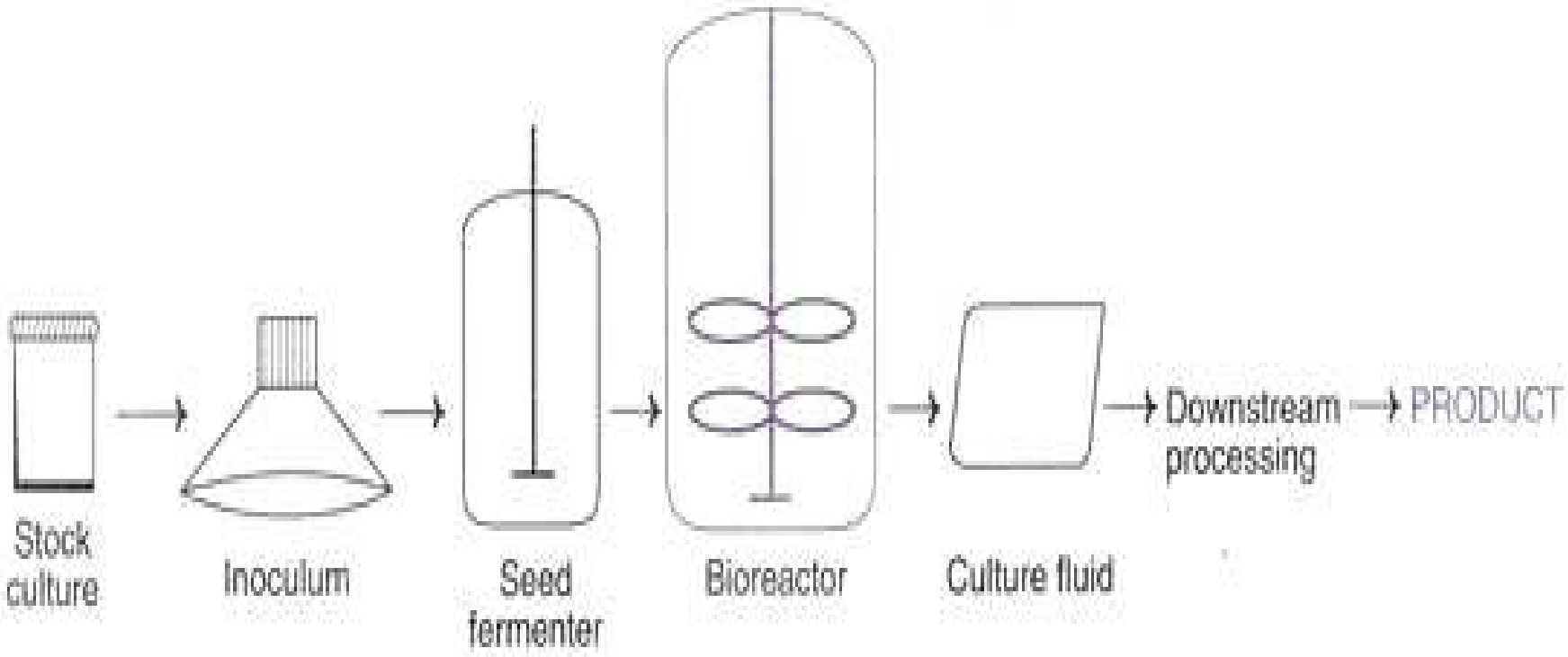


Fig. 19.16 : Diagrammatic representation of a generalized fermentation process.

UNIT I- FERMENTATION –ZYMOLOGY

(SCIENCE THAT DEALS WITH FERMENTATION)

II. FERMENTATION PROCESS

FERMENTER

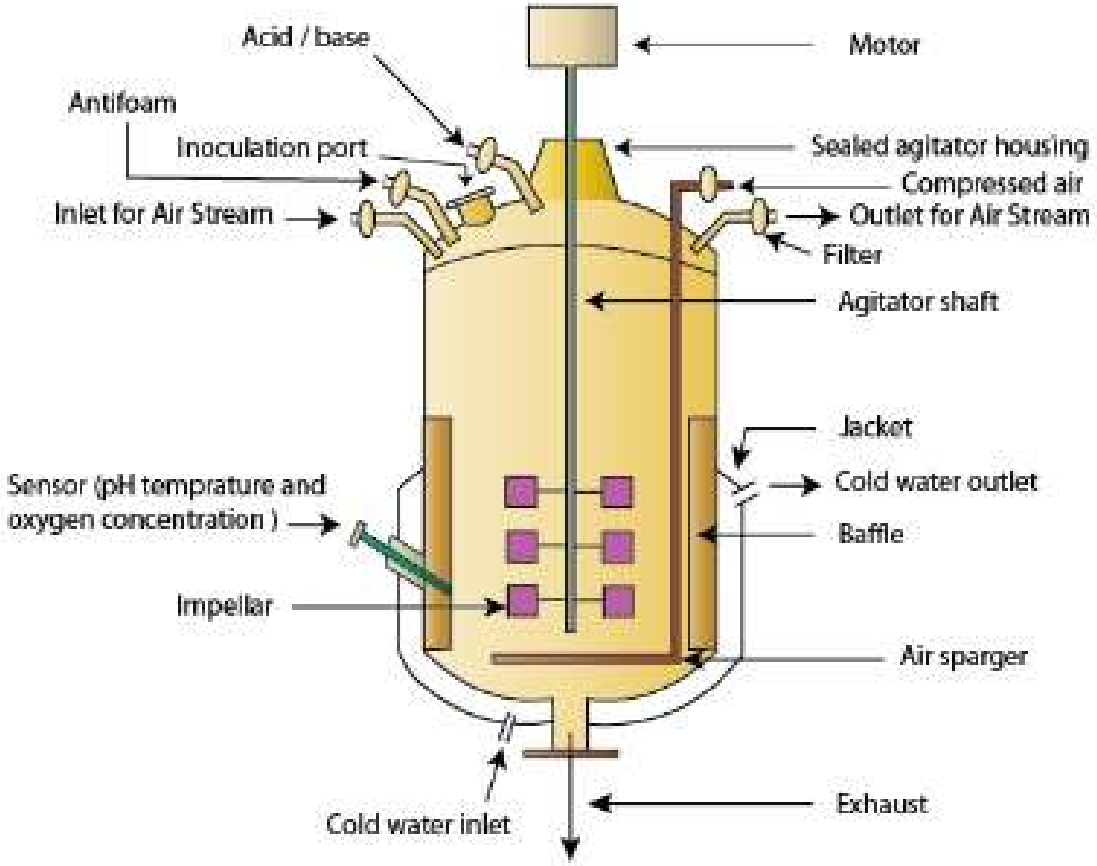
A fermenter is basically a device in which the substrate of low value is utilized by living cells or enzymes to generate a product of higher value.

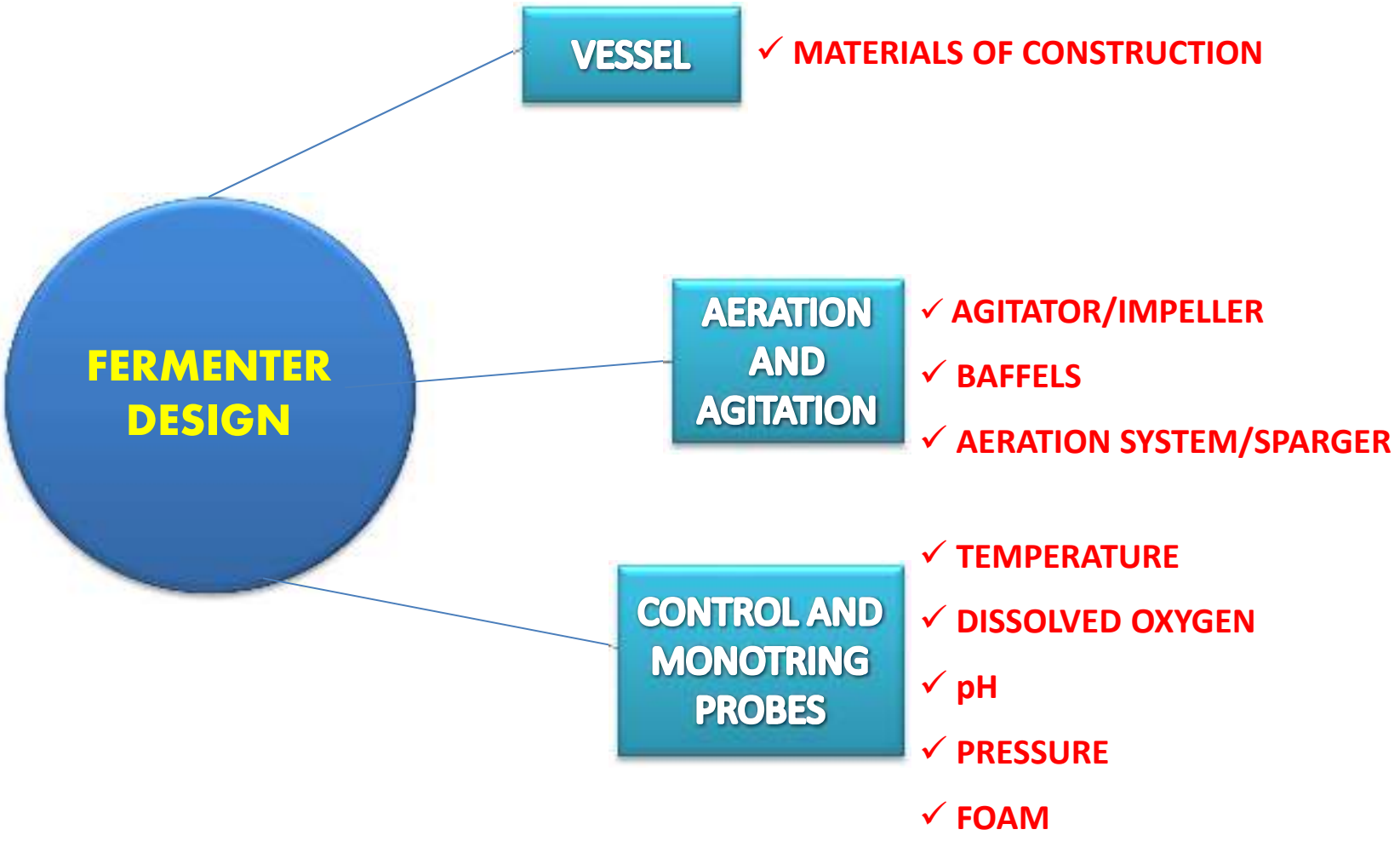
It is a containment system designed to give right environment for optimal growth and metabolic activity of the organism.

BASIC FUNCTION OF FERMENTER

• Provide a controlled environment for optimum product yield. • Power consumption should be minimum. • Facility for sampling should be provided. • Evaporation losses should be as low as possible. • It should have smooth internal surfaces and joints should be welded wherever possible • Provide adequate mixing and aeration for optimum growth and production of metabolites.

DIAGRAM OF A TYPICAL BIOREACTOR




FERMENTER DESIGN
VESSEL
✓ MATERIALS OF CONSTRUCTION
AERATION AND AGITATION
✓ AGITATOR/IMPELLER
✓ BAFFELS
✓ AERATION SYSTEM/SPARGER
CONTROL AND MONOTRING PROBES
✓ TEMPERATURE
✓ DISSOLVED OXYGEN
✓ pH
✓ PRESSURE
✓ FOAM

Various components of an ideal fermenter for batch process are:

S.N0	Part	Purpose
1	Top plate	Cover (made of steel)
2	Clamp	Top plate compressed onto vessel using clamp
3	Seal	Separates top plate from vessel (glass) to prevent air leakage
4	Vessel	Glass, jacketed, steel with ports for various outputs, inputs, probes etc.
5	Drive motor	Used to drive mixing shaft
6	Drive shaft	Mixes the medium evenly with its impeller
7	Impeller	Provides agitation
8	Baffles	Prevent sedimentation on sides and proper mixing
9	Sparger	Air supplier / after filtration via membranes – ensures efficient dispersal – by attached to impeller

S.N0	Part	Purpose
10	Exit gas cooler	Like condenser remove as much moisture as possible from exhaust
11	Inoculation needle	Port to add inoculum
12	Feed pumps	Regulates the flow rates of additives (medium, nutrients) variable speed
13	Peristaltic pumps	Fixed speed pumps – used for continuous sampling
14	Syringe pump	Using a syringe – mostly used in batch
15	Exit gas analysis	CO_2 analyzer, O_2 analyzer, mass spectrometer
16	Sample pipe	Through which samples are drawn
17	3 way inlet	To insert different probes

Monitoring and controlling parts of fermenter are:

S.N0	Part	Purpose
1	Pt100	Temperature sensor (platinum resistance electrode)
2	Foam probe	Kept above the medium level to sense foam formation
3	pH electrode	Senses pH
4	O_2 sensor	Monitors dissolved oxygen level
5	Heater pad	Directly heats the medium
6	Cold finger	After direct heating – used to cool the vessel contents (closed coil/pipe to pass cool water)
7	Rotameter	Variable air flow meter – indicates rate of air flow into vessel – attached to air sparger
8	Pressure valve	Attached to rotameter for safer operation
9	Air pump	Supply of air
	Peristaltic pump	To pump in medium, acids, bases, antifoam

1. AGITATOR

AGITATOR

- Synonym : impeller
- Mounted to a shaft through a bearing in the lid
- Driven by an external power source or direct drive
- Direct drive - action varied by using different impeller blades

MIXING OBJECTIVES IT ACHIEVE

- Bulk fluid & gas phase mixing
- Air dispersion
- O_2 transfer
- Heat transfer
- Suspension of solid particles
- Maintenance of uniform environment throughout the vessel

CLASSIFICATION

- Disc turbine
- Vaned disc
- Variable pitch open turbine
- Marine propellers

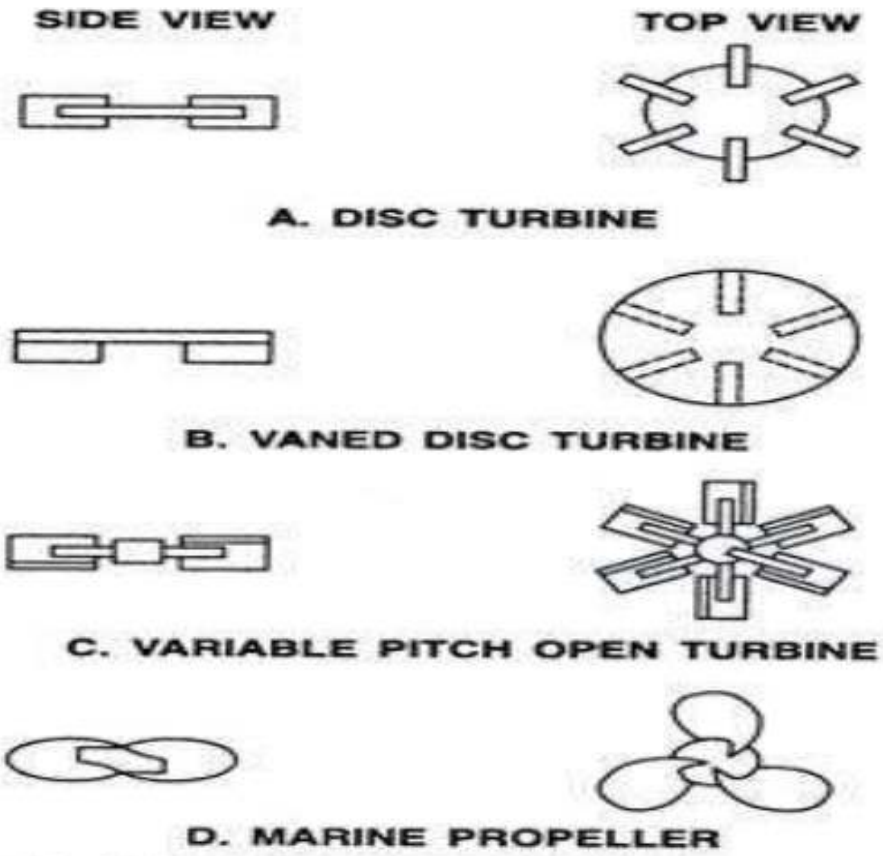


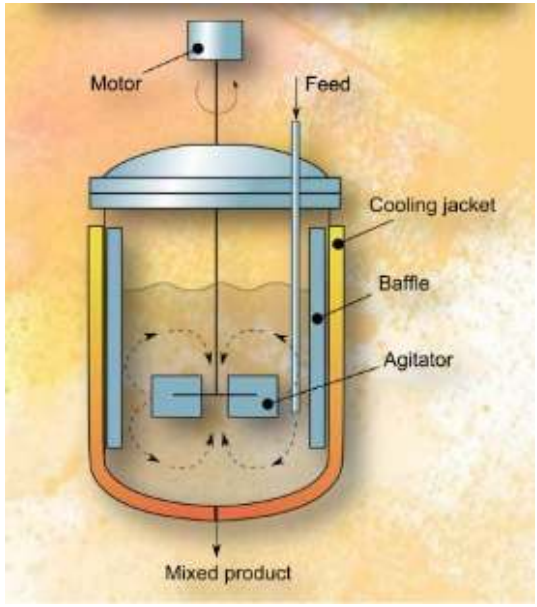
FIG. 14.1. Different types of agitators : **A.** disc turbine; **B.** vaned disc; **C.** open turbine, variable pitch; and **D.** marine propeller agitators.

2. BAFFLES

Baffles are metal strips that prevent vortex formation around the walls of the vessel.
These metal strips attached radially to the wall for every 1/10th of vessel diameter. Usually 4 baffles are present but when the vessel diameter is over 3dm3 around 6-8 baffles are used.

BAFFLES

- Metal strips
- $1/10^{th}$ of the vessel diameter
- Attached radially to wall
- 4 baffles (normal)
- Wider baffles - high agitation effect



- Narrower baffles – low agitation effect
- Can be attached with cooling coils
- Not found in lab scale fermentors
- Vertical baffles – increased aeration

3. AERATION SYSTEM (SPARGER)

Sparger is a device for introducing air into fermenter. Aeration provides sufficient oxygen for organism in the fermenter.

Fine bubble aerators must be used. Large bubbles will have less surface area than smaller bubbles which will facilitate oxygen transfer to a greater extent.

SPARGER TYPES

- Porous
- Orifice
- Nozzle

Porous

Orifice

Nozzle

1. Porous sparger: Made of sintered glass, ceramics or metal. It is used only in lab scale-non agitated vessel. The size of the bubble formed is 10-100 times larger than pore size.

There is a pressure drop across the sparger and the holes tend to be blocked by growth

which is the limitation of porous sparger.

2. Orifice sparger: Used in small stirred fermenter. It is a perforated pipe kept below the impeller in the form of crosses or rings. The size should be ~ ¾ of impeller diameter. Air holes drilled on the under surfaces of the tubes and the holes should be atleast 6mm diameter. This type of sparger is used mostly with agitation. It is also used with out agitation in some cases like yeast manufacture, effluent treatment and production of SCP.

3. Nozzle sparger: Mostly used in large scale. It is single open/partially closed pipe positioned centrally below the impeller. When air is passed through this pipe there is lower pressure loss and does not get blocked.

4. Combined sparger agitator: This is air supply via hallow agitator shaft. The air is emitted through holes in the disc or blades of agitator.

4. EXIT GAS COOLER

Similar to condenser, condenses the moisture from the exhaust gas in the fermenter. This removes as much moisture as possible from the gas leaving the fermenter and prevent excess fluid loss.

5. SEALING

Sealing between top plate and vessel is an important criteria to maintain airtight condition, aseptic and containment. Sealing have to be done between three types of surfaces *viz.* between glass-glass, glass- metal and metal-metal.

There are three types of sealing. They are *gasket*, *lipseal* and *'O' ring*. This sealing ensures tight joint in spite of expansion of vessel material during fermentation. The materials used for sealing may be fabric-nitryl or butyl rubbers. The seals should be changed after finite time. There are two way of sealing in O ring type simple sealing and double sealing with steam between two seals.

Optimization of Fermentation Process

- ✓The optimization of fermentation process is based on usage of metabolic and enzymatic activities of microbial organisms, as they are involved in production of desired products.
- ✓The main objective of designing and optimization is to study the previous effects and interactions, to estimate the metabolic activities of microbial organisms.
- ✓Optimization can be done by simple approach and is used to continuously improve and existing fermentation process.
- ✓For optimal designing of fermentation process, computer generated designs are useful in fermentation when certain trails cannot be run due to imbalanced physical or operational factors.
- ✓Industrial fomenters designed to provide best possible growth and biosynthetic environment for the culture.

- ✓Suitable grade of stainless steel is used for the construction of ideal fermenter, as it is more reliable, relatively stronger than ordinary steel and it has high mechanical strength and as well as corrosion resistance
- ✓Since most industrial fomenters utilize pure culture, the vessel must have some provisions to prevent or control the growth of unuseful organisms.
- ✓Traditional fomenters are cylindrical shape with dome shaped surfaces, the fermenter vessel surrounded by cooling jacket, sparger attached at the bottom, the stirrer shaft connected to a motor which can rotate the shaft and followed by impellers.

The vessel connected different control ports like temperature probe, pH probe, anti foam probe, feed inlet, culture inlet and DO sensors.

- ✓The vessel has a provision of inoculation and sampling inlets and also charge and discharge pipes.

- ✓The fermenter should work at high temperature, so that the vessel must be strong enough to withstand the changes occurred in temperature and pressure by forced agitation.
- ✓During operation, the sterilization of fomenters protocols are strictly adhered for good fermentation process.
- ✓Sterilization approaches are- insitu and continuous heat sterilization:
  - ✓**Insitu -** the whole system is heated to 120^0 C
  - ✓**Contious sterilization-** the medium rapidly heated to 140^0 C for short period by injection steam.

- ✓ The sterilized microbes / culture is added to culture media and the size of Inoculum is generally 1-10% of the total volume of medium needed.
- ✓ Proper agitation and aeration must be provided to maintain uniform environment in the vessel . Sparger used for aeration, by air lift system or stirred aeration system. Maintaining of Control parameters is very essential for providing aseptic and uniform environment.
- ✓ It is necessary to control the pH changes of the media by adding alkali or acids.
- ✓Temperature control is very important for good fermentation, as rapid multiplication of culture can generate a large amount of heat and it should removed from vessel through cooling jacket.
- ✓Some culture requires high temperature, for them it is necessary to pass steam to rise the temperature for their metabolism.

- ✓ Dissolved Oxygen is sparingly soluble in water, it should be 0.0084gm/lit at 25^0C.
- ✓ After completion of fermentation process, the fermenter is cleaned, sterilized and make it ready for next batch fermentation process.
- ✓Typical control parameters range-

Temperature –	8 - 60^0C
Stability -	> 98%
Air	0-6lit/min
pH-	2-12
Agitation-	1000rpm
CO2-	0-100%
Pressure-	2000mbr

Fermenters - Types

Fermenters are bioreactors have a cylindrical shape in which the process of fermentation is carried out. There are different types of fermenters used by industries for the production of desired products.

The main function of a fermenter is to provide a controlled environment for the growth of microbes/culture. In designing and constructing a fermenter a number of points must be considered:

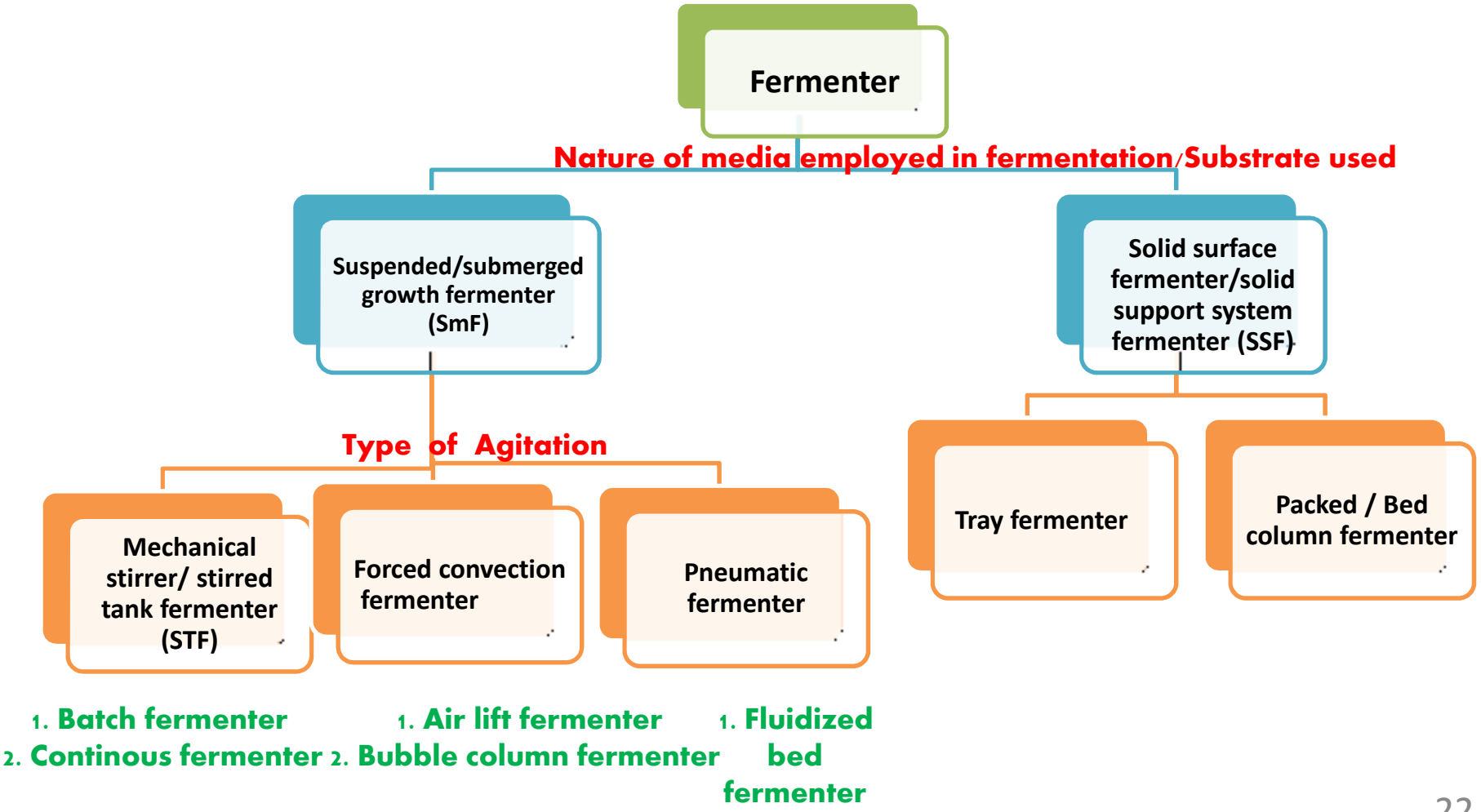
- ✓**The vessel should be capable of being operated aseptically for a number of days and should be reliable in long term operation.**
- ✓**Adequate aeration and agitation should be provided to meet the metabolic requirements of the micro organisms. However, the mixing should not cause damage to the organism.**
- ✓**Power consumption should be as low as possible.**
- ✓**A system of temperature control & pH should be provided.**
- ✓**Sampling facilities should be provided.**
- ✓**The vessel should be designed to require the minimal use of labour in operation, harvesting, cleaning and maintenance.**

According to sizes:

Small lab and research fermenter – **1-50L**

Pilot plant fermenter - **50-1000L**

Large size industrial production scale fermenter- **more than 1000l**


Fermenter
Nature of media employed in fermentation/Substrate used
Suspended/submerged growth fermenter (SmF)
Solid surface fermenter/solid support system fermenter (SSF)
Type of Agitation
Mechanical stirrer/ stirred tank fermenter (STF)
Forced convection fermenter
Pneumatic fermenter
Tray fermenter
Packed / Bed column fermenter
1. Batch fermenter
2. Continous fermenter
1. Air lift fermenter
2. Bubble column fermenter
1. Fluidized bed fermenter

I. Submerged fermenters- in liquid media

In this type of fermenters, the cells are freely suspended in the culture medium, to grow and formation of product.

The agitation in media provides movements for the mixing of air / gases, allow better contact between cultured cells and substrate.

1. **Mechanical stirrer/ stirred tank fermenter (STF)**
2. **Forced convection fermenter**
3. **Pneumatic fermenter**

The common substrates used in submerged fermentation system are soluble sugars, vegetable juices, and sewage water

II. Supported growth fermenters---process occuring in the absence of water

These are also called surface culture fermenter system. In this type of fermenter, vegetative microbial cells are cultured on a support or surface, inside the culture media. The cells are not freely suspended in media.

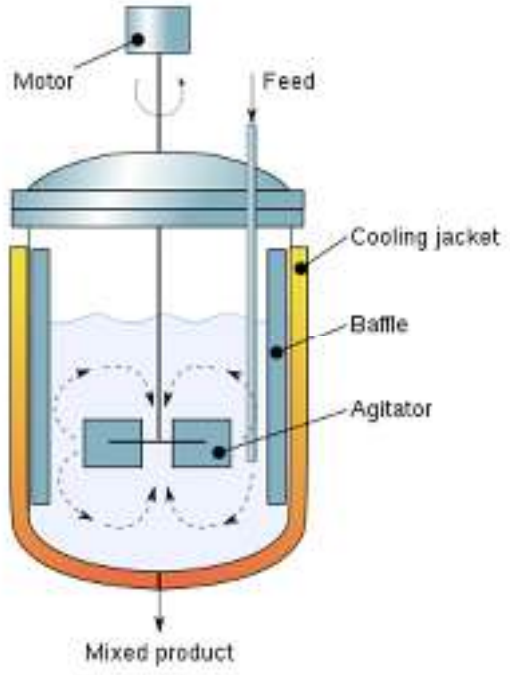
The main advantage of this is, better aeration, high product yield and easier product isolation or easy downstream process

1. **Tray fermenter**
2. **Packed / Bed column fermenter**

microbial cells utilized solid nutrient-rich waste materials such as grains, Bran, Bagasse, and paper pulp.

1. Mechanical stirrer/ stirred tank fermenter (STF)

They follow the structure of a basic fermenters, as they contains all basic parts of a typical fermenter, and a mechanical stirrer in centre to agitate the media filled inside fermenter. This agitation is necessary for mixing of filled material, heat transfer, mass transfer, and appropriate mixing if gases in media.



These are upright cylindrical shaped fermenters, and the selected volume should be more than 30-50% of the volume liquid or the media, which is to be filled in it.

Because the upper head space is required for gases and foam which is produced during fermentation.

Advantages : There are many advantages of STRs over other types. These include the efficient gas transfer to growing cells, good mixing of the contents and flexible operating conditions, besides the commercial availability of the bioreactors.

2. Forced Convection Fermenter

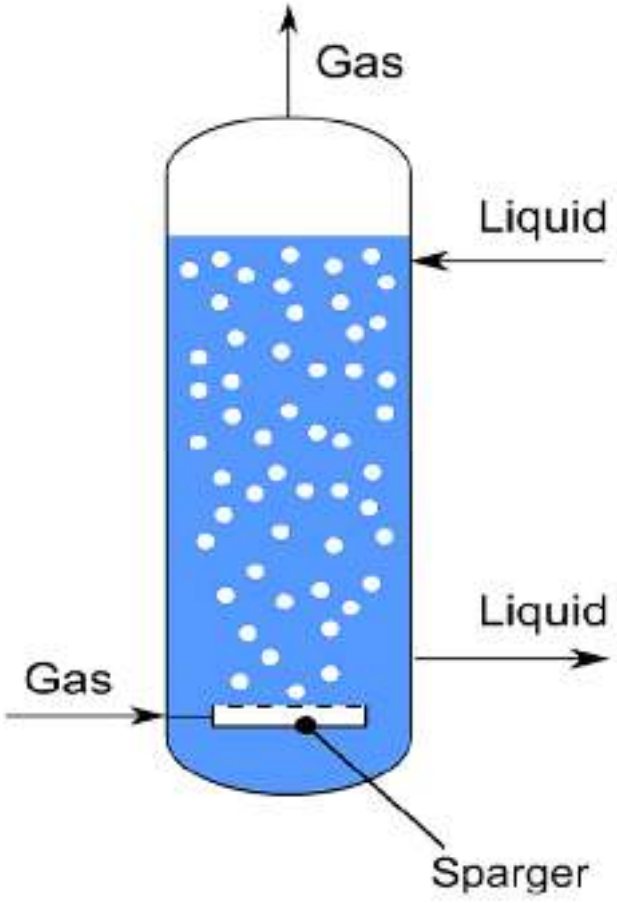
In these types of fermenters, the agitation is achieved through the movement of liquid and gas, by using pump. No mechanical impellers are employed for liquid movement.

They are two types: **A. Bubble Column Fermenter**
B. Air Lift Fermenter

A. Bubble Column fermenter or Sparged gas fermenter

These are simple types of convection fermenters. The height is three times of the diameter. The gas, usually air is sparged or introduce through the spargers, fitted at the bottom of the fermenter.

This gas / air moves upward and creates agitation in the medium. The jets of sparger are evenly distributed all over bottom to create uniform agitation throughout the media. These fermenters has no mechanical impellers, so they need less energy.



B. Air Lift Fermenter

The height is around 10 times than the diameter of these fermenter vessels. The movement of media is achieved by change in its density.

The gas is introduced at a specific area called riser. The presence of gas in the form of bubbles, reduces the density of liquid media, and this less density media moves upward.

At the top, the gas is gets separated from liquid, and due to this, liquid gets higher density, and moved downward. This creates a cyclic movement in liquid, cause agitation.

Airlift fermenter

- 1)Concentric draft tube airlift fermenter

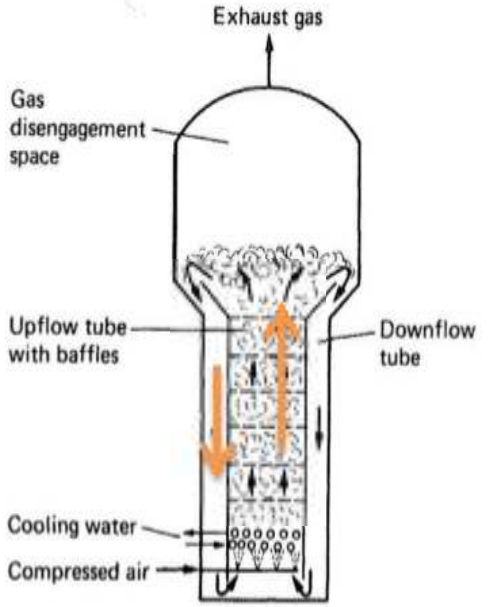


FIG. 7.47b. Air-lift fermenter with inner loop (Smith, 1980).

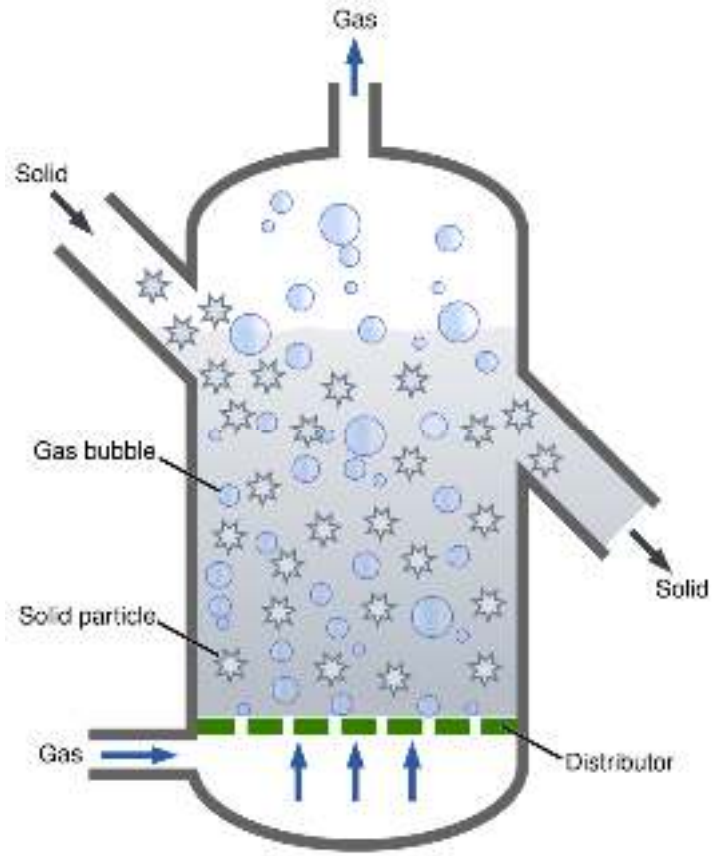
3. Pneumatic Fermenter: -

Fluidized bed fermenter

These are similar to column reactors, but upper part is more wider, to minimize the liquid flow speed. The microbial cells are grown on any spherical support like glass balls or any porous material, are suspended in liquid media.

From the bottom, the air gas introduce in such a way that it makes the balls to float inside liquid medium. The flow of gas and the density of support material is adjusted in such a way that the balls are not thrown outside. The liquid, and the gas pressure not such low that the balls are settled down at the bottom of the reactor.

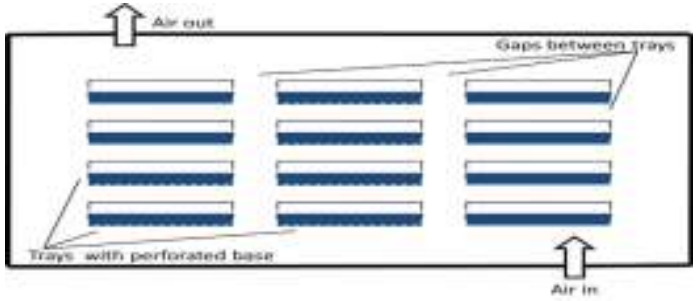
This type of reactor provides better contact of gas with the microbial cells.

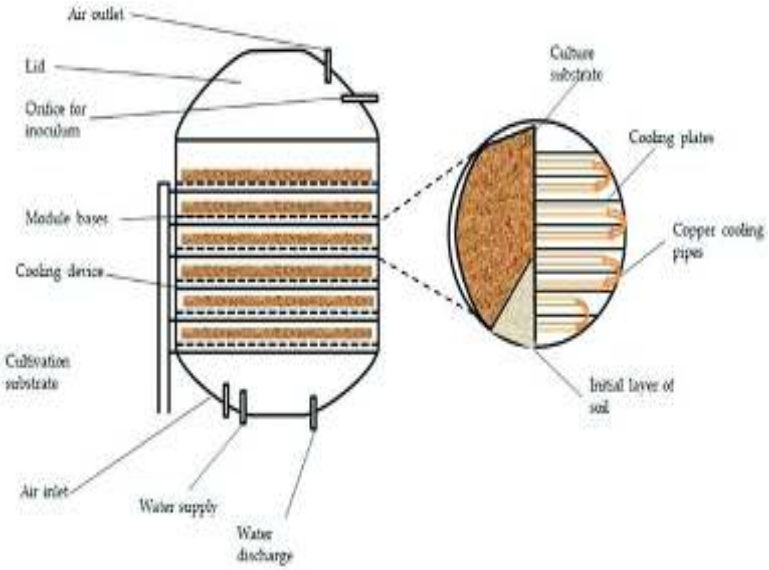


II. Supported growth fermenters

1. Tray fermenter:

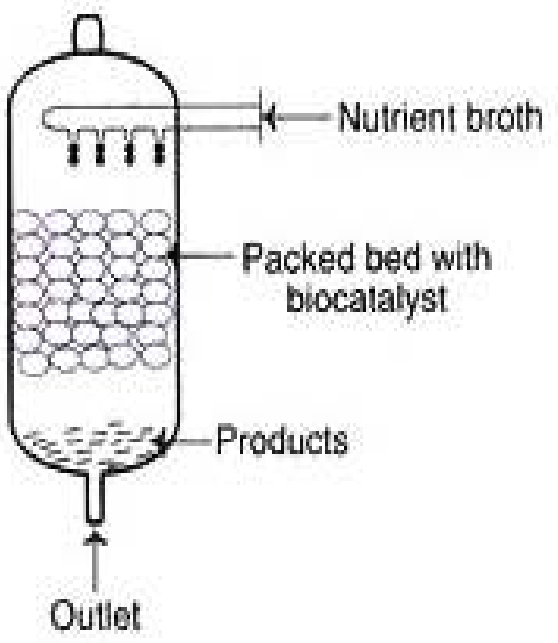
Tray fermenters are the simplest of all types of fermenters and may be wooden, metallic (aluminum or iron). If iron is used, it should be painted to avoid corrosion. The bottom is perforated in such a way that it holds substrate and allows aeration of the under surface of the substrate. Usually, trays are arranged one above the other with a suitable gap between trays. The fermentation is carried out in a chamber where a controlled humid atmosphere is created. The temperature of the fermenting substrate is controlled by circulating warm or cool air as necessary.





2. Packed / Bed column fermenter

A bed of solid particles, with biocatalysts on or within the matrix of solids, packed in a column constitutes a packed bed bioreactor. The solids used may be porous or non-porous gels, and they may be compressible or rigid in nature. A nutrient broth flows continuously over the immobilised biocatalyst. The products obtained in the packed bed bioreactor are released into the fluid and removed. While the flow of the fluid can be upward or downward, down flow under gravity is preferred.

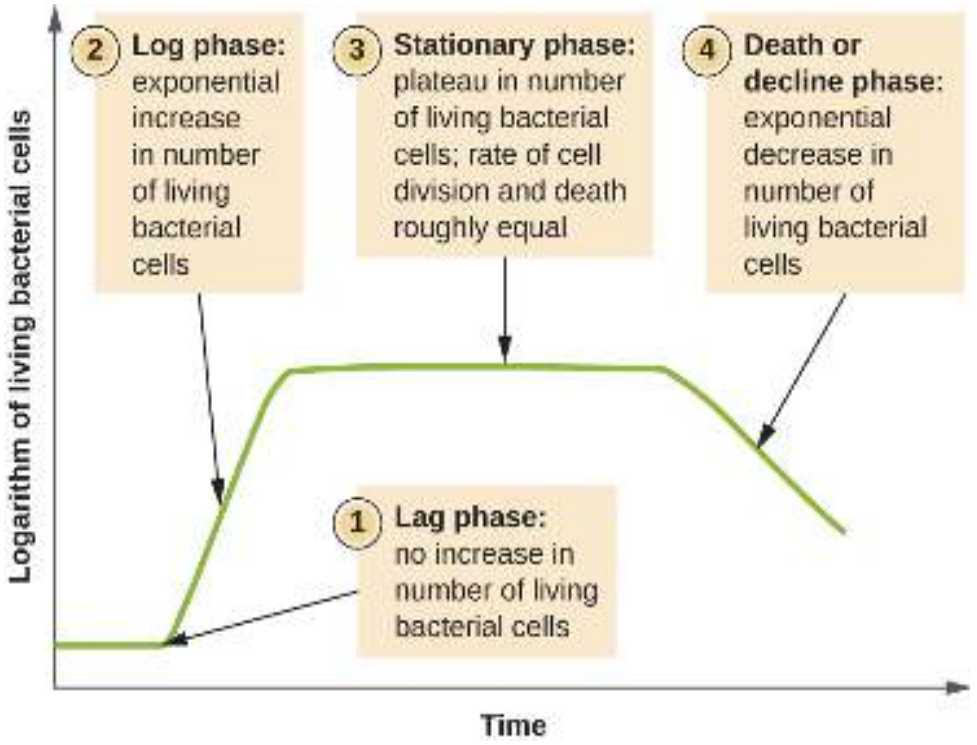


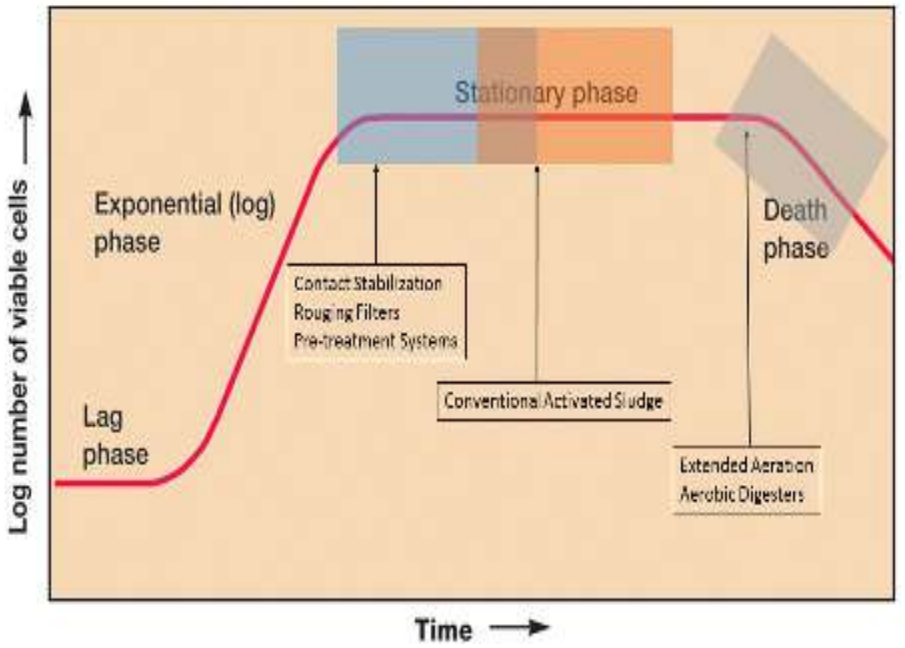
The concentration of the nutrients (and therefore the products formed) can be increased by increasing the flow rate of the nutrient broth. Because of poor mixing, it is rather difficult to control the pH of packed bed bioreactors by the addition of acid or alkali. However, these bioreactors are preferred for bioprocessing technology involving product-inhibited reactions. The packed bed bioreactors do not allow accumulation of the products to any significant extent.

The packed bed reactors are widely used with immobilized cells. Several modifications such as tapered beds to reduce the pressure drop across the length of the reactor, inclined bed, horizontal bed, rotary horizontal reactors have been tried with limited success.

FERMENTATION TYPES

Microbial - Growth Curve



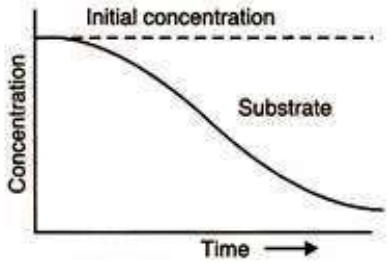


1. Batch Fermentation
2. Continuous Fermentation
3. Fed Batch Fermentation

1. Batch Fermentation

A batch fermentation is a closed culture system, because initial and limited amount of sterilized nutrient medium is introduced into the fermenter. The medium is inoculated with a suitable microorganism and incubated for a definite period for fermentation to proceed under optimal physiological conditions. Oxygen in the form of air, an antifoam agent and acid or base, to control the pH, are being added during the course of fermentation process.

During the course of incubation, the cells of the microorganism undergo multiplication and pass through different phases of growth and metabolism due to which there will be change in the composition of culture medium, the biomass and metabolites. The fermentation is run for a definite period or until the nutrients are exhausted. The culture broth is harvested and the product is separated.



The used medium along with cells of microorganism and the product is drawn out from the fermenter. When the desired product is formed in optimum quantities, the product is separated from the microorganism and purified later on

Lag phase -adapt to their surroundings

Exponential growth - grow in numbers

Stationary phase -stop growing

Death phase

Merits:

(a) The possibility of contamination and mutation is very less.

(b) Simplicity of operation and reduced risk of contamination.

Demerits:

(a) For every fermentation process, the fermenter and other equipment are to be cleaned and sterilized.

(b) Only fraction of each batch fermentation cycle is productive.

(c) It is useful in fermentation with high yield per unit substratum and cultures that can tolerate initial high substrate concentration.

(d) It can be run in repeated mode with small portion of the previous batch left in the fermenter for inoculum.

(e) Use of fermenter is increased by eliminating turn round time or down time.

(f) Running costs are greater for preparing and maintaining stock cultures.

(g) Increased, frequency of sterilization may also cause greater stress on instrumentation and probes.

(h) Fresh sterilized medium and pure culture are to be made for every fermentation process.

(i) Yield of the desired product may also vary.

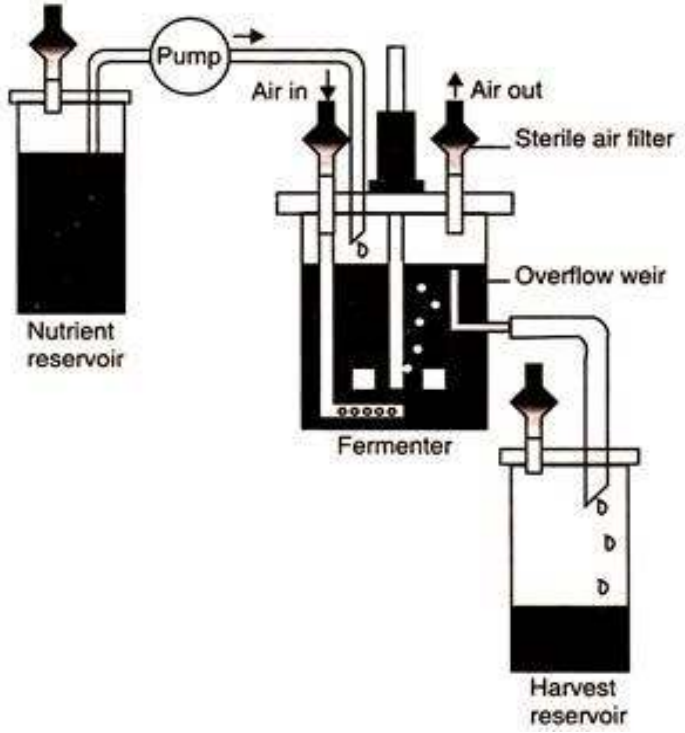
(j) There will be a non-productive period of shutdown between one batch productive fermentation to the other,

(k) More personal are required.

2. Continuous Fermentation:

It is a closed system of fermentation, run for indefinite period. In this method, fresh nutrient medium is added continuously or intermittently to the fermenter and equivalent amount of used medium with microorganisms is withdrawn continuously or intermittently for the recovery of cells or fermentation products.

As a result, volume of the medium and concentration of nutrients at optimum level are being maintained. This has been operated in an automatic manner. The continuous fermenter has its maximum use that take long time to reach high productivity, reduces down time and lowers the operating costs.



In continuous mode, starting medium and inoculum are added to the fermenter. After the culture is grown the fermenter is fed with nutrients and broth is withdrawn at the same rate maintaining a constant volume of broth in the fermenter. In continuous mode with cell cycle, the cell mass is returned to the fermenter using micro filtrations with bacteria or screens with fungal mycelium.

Merits:

1. The fermenter is continuously used with little or no shutdown time.
2. Only little quantity of initial inoculum is needed and there is no need of additional inoculum.
3. It facilitates maximum and continuous production of the desired product.
4. There is optimum utilization of even slow utilizable substances like hydrocarbons.

Demerits:

1. Possibility of contamination and mutation because of prolonged incubation and continuous fermentation, are more.
2. Possibility of wastage of nutrient medium because of continuous withdrawal for product isolation.
3. The process becomes more complex and difficult to accomplish when the desired products are antibiotics rather than a microbial cells.
4. Lack of knowledge of dynamic aspects of growth and synthesis of product by microorganism used in fermentation.

Applications:

Continuous culture fermentation has been used for the production of single cell protein, antibiotics, organic solvents, starter cultures.

3. Fed Batch Fermentation:

It is a modification to the batch fermentation. In this process substrate is added periodically in instalments as the fermentation progresses, due to which the substratum is always at an optimal concentration. This is essential as some secondary metabolites are subjected to catabolite repression by high concentration of either glucose, or other carbohydrate or nitrogen compounds present in the medium.

For this reason, the critical elements of the nutrient medium are added in low amount in the beginning of the fermentation and these substrates continue to be added in small doses during the production phase. This method is generally employed for the production of substances such as penicillin. Yoshida (1973) introduced this term for the first time for feeding the substrates to the medium as the nutrients are exhausted, so as to maintain the nutrients at an optimum level.

UNIT I- FERMENTATION –ZYMOLOGY

(SCIENCE THAT DEALS WITH FERMENTATION)

III. DOWNSTRREAM PROCESS

DOWNSTREAM PROCESS

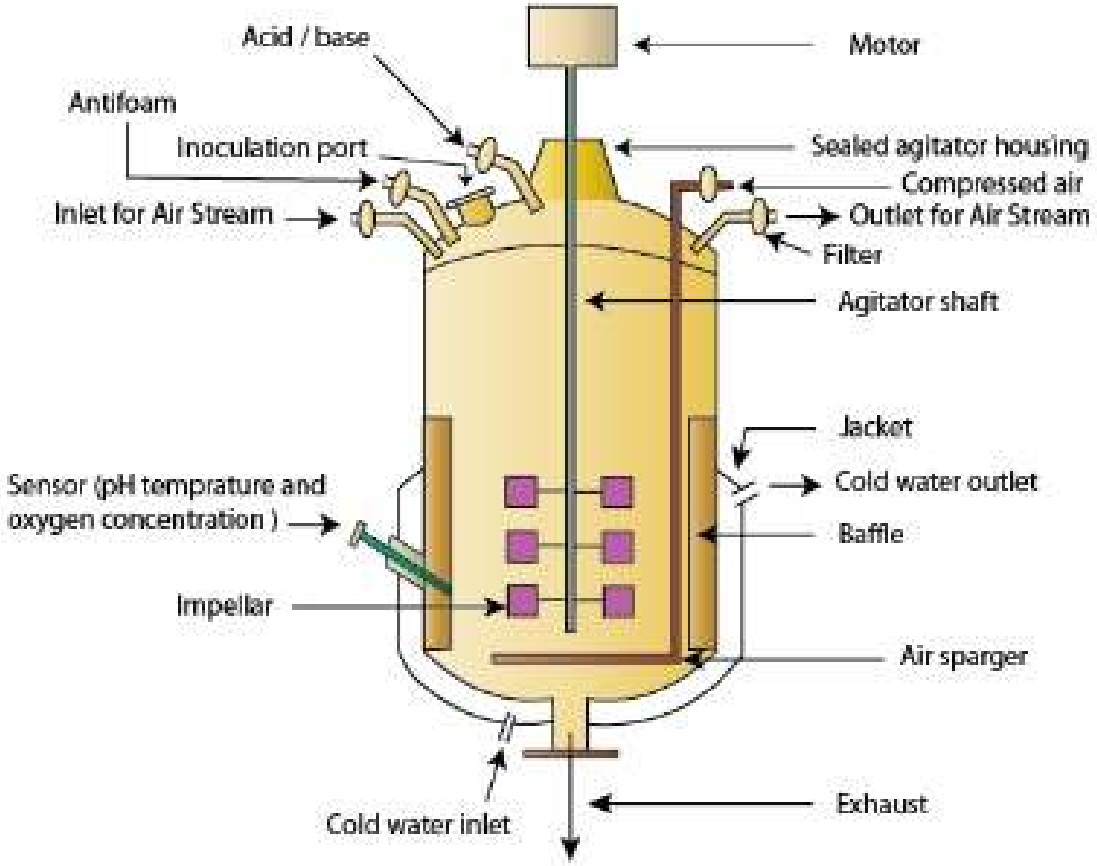
The downstream processing deals with the following:

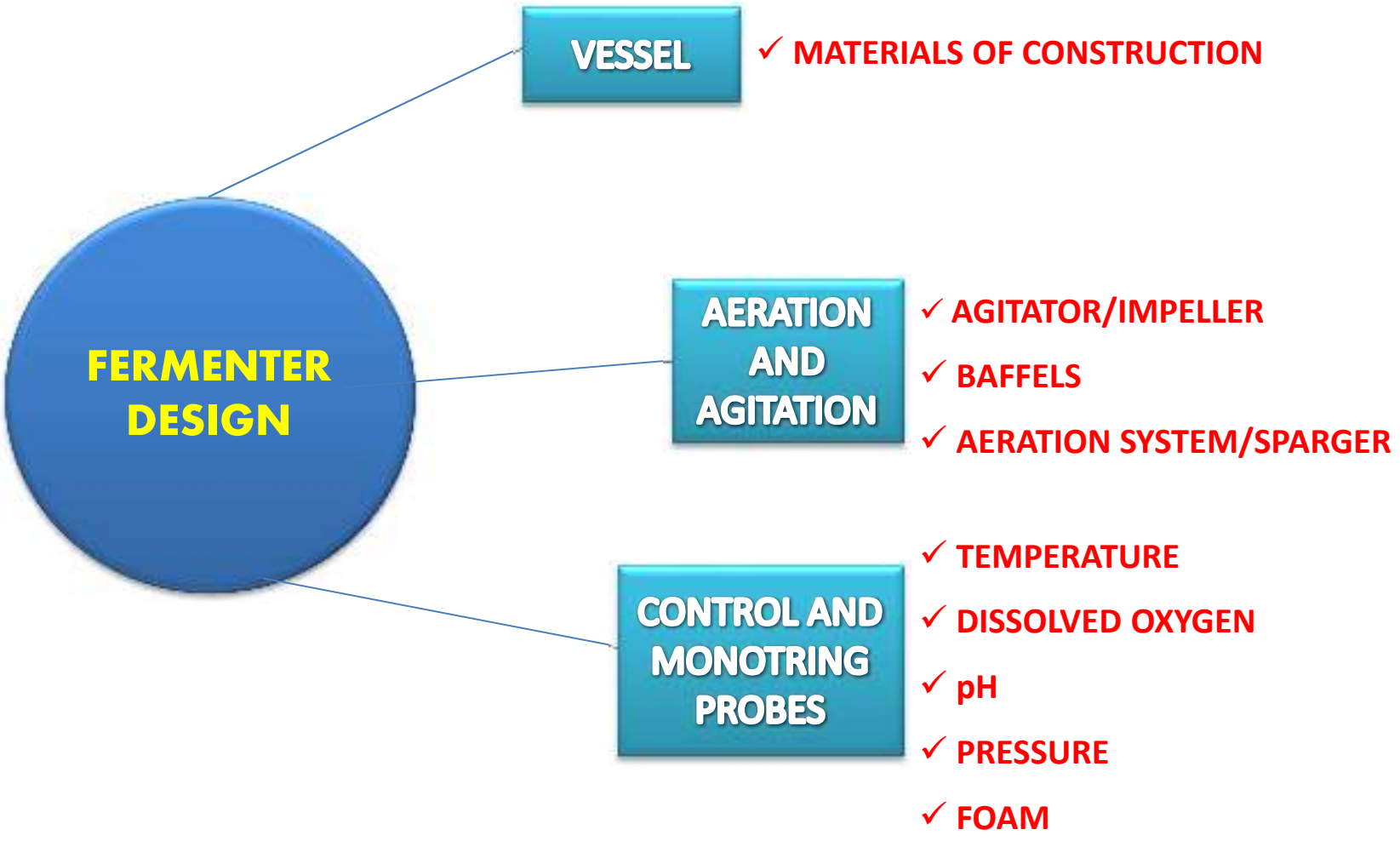
• Post harvest product recovery- clarification, concentration, purification, polishing and formulation till packaging of the desired product.

What are the basic strategies of down stream processing?

- Separation of insolubles (cells, debris, precipitates etc)
- Extraction (removal of molecules that are dissimilar to product , removal of water etc)
- Purification (removal of molecules similar to product)
- Polishing (packing in a form easy to transport, handle and give stability to product)

DIAGRAM OF A TYPICAL BIOREACTOR




FERMENTER DESIGN
VESSEL
✓ MATERIALS OF CONSTRUCTION
AERATION AND AGITATION
✓ AGITATOR/IMPELLER
✓ BAFFELS
✓ AERATION SYSTEM/SPARGER
CONTROL AND MONOTRING PROBES
✓ TEMPERATURE
✓ DISSOLVED OXYGEN
✓ pH
✓ PRESSURE
✓ FOAM

Various components of an ideal fermenter for batch process are:

S.N0	Part	Purpose
1	Top plate	Cover (made of steel)
2	Clamp	Top plate compressed onto vessel using clamp
3	Seal	Separates top plate from vessel (glass) to prevent air leakage
4	Vessel	Glass, jacketed, steel with ports for various outputs, inputs, probes etc.
5	Drive motor	Used to drive mixing shaft
6	Drive shaft	Mixes the medium evenly with its impeller
7	Impeller	Provides agitation
8	Baffles	Prevent sedimentation on sides and proper mixing
9	Sparger	Air supplier / after filtration via membranes – ensures efficient dispersal – by attached to impeller

S.N0	Part	Purpose
10	Exit gas cooler	Like condenser remove as much moisture as possible from exhaust
11	Inoculation needle	Port to add inoculum
12	Feed pumps	Regulates the flow rates of additives (medium, nutrients) variable speed
13	Peristaltic pumps	Fixed speed pumps – used for continuous sampling
14	Syringe pump	Using a syringe – mostly used in batch
15	Exit gas analysis	CO_2 analyzer, O_2 analyzer, mass spectrometer
16	Sample pipe	Through which samples are drawn
17	3 way inlet	To insert different probes

Monitoring and controlling parts of fermenter are:

S.N0	Part	Purpose
1	Pt100	Temperature sensor (platinum resistance electrode)
2	Foam probe	Kept above the medium level to sense foam formation
3	pH electrode	Senses pH
4	O_2 sensor	Monitors dissolved oxygen level
5	Heater pad	Directly heats the medium
6	Cold finger	After direct heating – used to cool the vessel contents (closed coil/pipe to pass cool water)
7	Rotameter	Variable air flow meter – indicates rate of air flow into vessel – attached to air sparger
8	Pressure valve	Attached to rotameter for safer operation
9	Air pump	Supply of air
	Peristaltic pump	To pump in medium, acids, bases, antifoam

1. AGITATOR

AGITATOR

- Synonym : impeller
- Mounted to a shaft through a bearing in the lid
- Driven by an external power source or direct drive
- Direct drive - action varied by using different impeller blades

MIXING OBJECTIVES IT ACHIEVE

- Bulk fluid & gas phase mixing
- Air dispersion
- O_2 transfer
- Heat transfer
- Suspension of solid particles
- Maintenance of uniform environment throughout the vessel

CLASSIFICATION

- Disc turbine
- Vaned disc
- Variable pitch open turbine
- Marine propellers

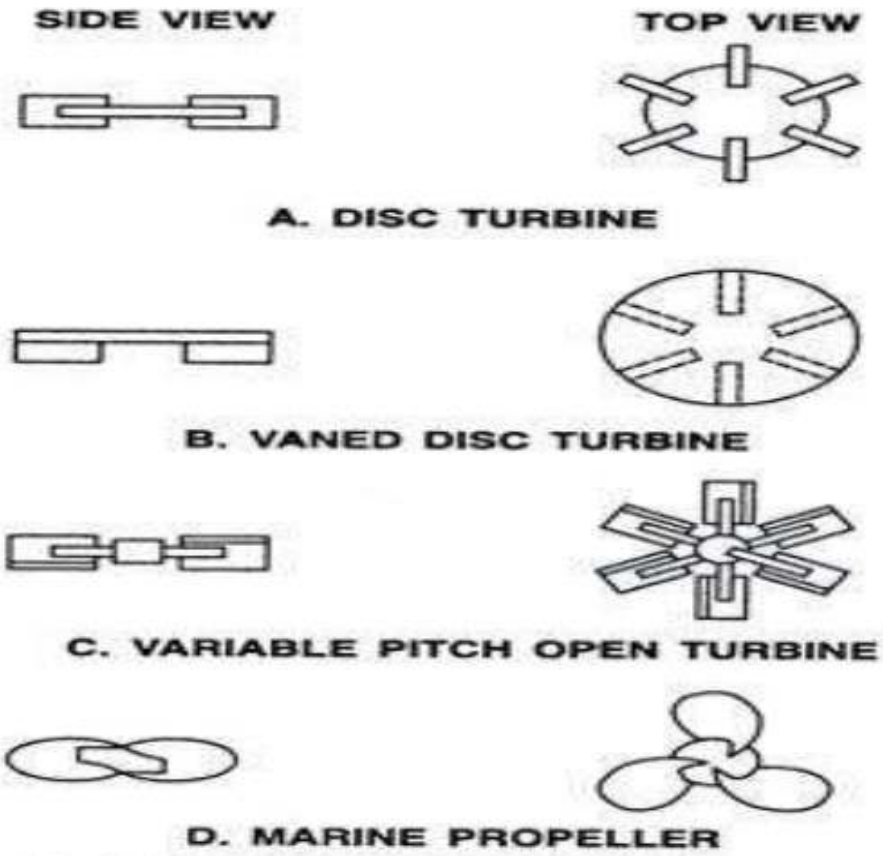


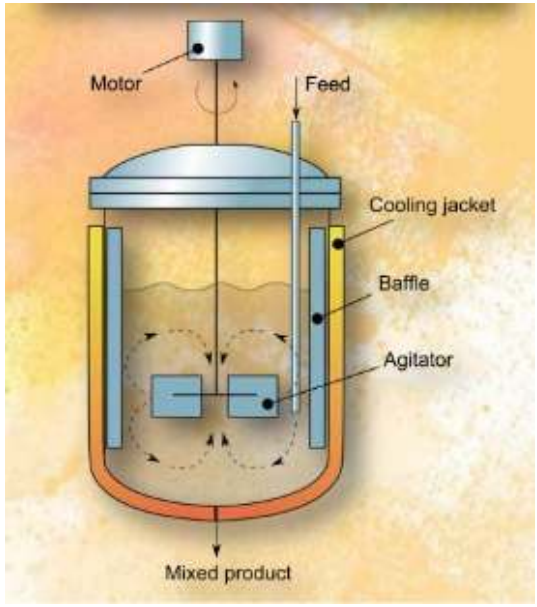
FIG. 14.1. Different types of agitators : **A.** disc turbine; **B.** vaned disc; **C.** open turbine, variable pitch; and **D.** marine propeller agitators.

2. BAFFLES

Baffles are metal strips that prevent vortex formation around the walls of the vessel.
These metal strips attached radially to the wall for every 1/10th of vessel diameter. Usually 4 baffles are present but when the vessel diameter is over 3dm3 around 6-8 baffles are used.

BAFFLES

- Metal strips
- $1/10^{th}$ of the vessel diameter
- Attached radially to wall
- 4 baffles (normal)
- Wider baffles - high agitation effect



- Narrower baffles – low agitation effect
- Can be attached with cooling coils
- Not found in lab scale fermentors
- Vertical baffles – increased aeration

3. AERATION SYSTEM (SPARGER)

Sparger is a device for introducing air into fermenter. Aeration provides sufficient oxygen for organism in the fermenter.

Fine bubble aerators must be used. Large bubbles will have less surface area than smaller bubbles which will facilitate oxygen transfer to a greater extent.

SPARGER TYPES

- Porous
- Orifice
- Nozzle

Porous

Orifice

Nozzle

1. Porous sparger: Made of sintered glass, ceramics or metal. It is used only in lab scale-non agitated vessel. The size of the bubble formed is 10-100 times larger than pore size.

There is a pressure drop across the sparger and the holes tend to be blocked by growth

which is the limitation of porous sparger.

2. Orifice sparger: Used in small stirred fermenter. It is a perforated pipe kept below the impeller in the form of crosses or rings. The size should be ~ ¾ of impeller diameter. Air holes drilled on the under surfaces of the tubes and the holes should be atleast 6mm diameter. This type of sparger is used mostly with agitation. It is also used with out agitation in some cases like yeast manufacture, effluent treatment and production of SCP.

3. Nozzle sparger: Mostly used in large scale. It is single open/partially closed pipe positioned centrally below the impeller. When air is passed through this pipe there is lower pressure loss and does not get blocked.

4. Combined sparger agitator: This is air supply via hallow agitator shaft. The air is emitted through holes in the disc or blades of agitator.

4. EXIT GAS COOLER

Similar to condenser, condenses the moisture from the exhaust gas in the fermenter. This removes as much moisture as possible from the gas leaving the fermenter and prevent excess fluid loss.

5. SEALING

Sealing between top plate and vessel is an important criteria to maintain airtight condition, aseptic and containment. Sealing have to be done between three types of surfaces *viz.* between glass-glass, glass- metal and metal-metal.

There are three types of sealing. They are *gasket*, *lipseal* and *'O' ring*. This sealing ensures tight joint in spite of expansion of vessel material during fermentation. The materials used for sealing may be fabric-nitryl or butyl rubbers. The seals should be changed after finite time. There are two way of sealing in O ring type simple sealing and double sealing with steam between two seals.

Optimization of Fermentation Process

- ✓The optimization of fermentation process is based on usage of metabolic and enzymatic activities of microbial organisms, as they are involved in production of desired products.
- ✓The main objective of designing and optimization is to study the previous effects and interactions, to estimate the metabolic activities of microbial organisms.
- ✓Optimization can be done by simple approach and is used to continuously improve and existing fermentation process.
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- ✓The vessel has a provision of inoculation and sampling inlets and also charge and discharge pipes.

- ✓The fermenter should work at high temperature, so that the vessel must be strong enough to withstand the changes occurred in temperature and pressure by forced agitation.
- ✓During operation, the sterilization of fomenters protocols are strictly adhered for good fermentation process.
- ✓Sterilization approaches are- insitu and continuous heat sterilization:
  - ✓**Insitu -** the whole system is heated to 120^0 C
  - ✓**Contious sterilization-** the medium rapidly heated to 140^0 C for short period by injection steam.

- ✓ The sterilized microbes / culture is added to culture media and the size of Inoculum is generally 1-10% of the total volume of medium needed.
- ✓ Proper agitation and aeration must be provided to maintain uniform environment in the vessel . Sparger used for aeration, by air lift system or stirred aeration system. Maintaining of Control parameters is very essential for providing aseptic and uniform environment.
- ✓ It is necessary to control the pH changes of the media by adding alkali or acids.
- ✓Temperature control is very important for good fermentation, as rapid multiplication of culture can generate a large amount of heat and it should removed from vessel through cooling jacket.
- ✓Some culture requires high temperature, for them it is necessary to pass steam to rise the temperature for their metabolism.

- ✓ Dissolved Oxygen is sparingly soluble in water, it should be 0.0084gm/lit at 25^0C.
- ✓ After completion of fermentation process, the fermenter is cleaned, sterilized and make it ready for next batch fermentation process.
- ✓Typical control parameters range-

Temperature –	8 - 60^0C
Stability -	> 98%
Air	0-6lit/min
pH-	2-12
Agitation-	1000rpm
CO2-	0-100%
Pressure-	2000mbr

Fermenters - Types

Fermenters are bioreactors have a cylindrical shape in which the process of fermentation is carried out. There are different types of fermenters used by industries for the production of desired products.

The main function of a fermenter is to provide a controlled environment for the growth of microbes/culture. In designing and constructing a fermenter a number of points must be considered:

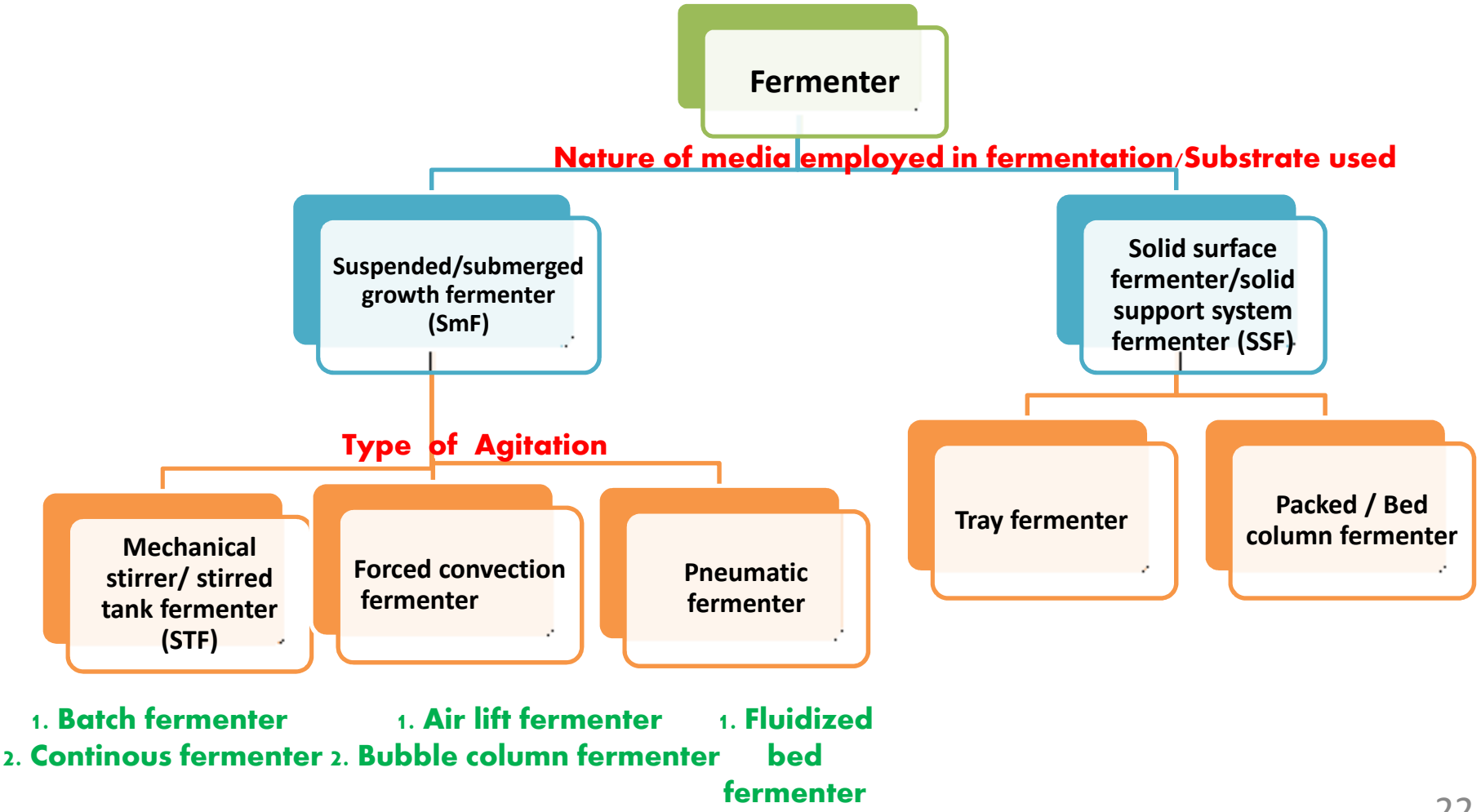
- ✓**The vessel should be capable of being operated aseptically for a number of days and should be reliable in long term operation.**
- ✓**Adequate aeration and agitation should be provided to meet the metabolic requirements of the micro organisms. However, the mixing should not cause damage to the organism.**
- ✓**Power consumption should be as low as possible.**
- ✓**A system of temperature control & pH should be provided.**
- ✓**Sampling facilities should be provided.**
- ✓**The vessel should be designed to require the minimal use of labour in operation, harvesting, cleaning and maintenance.**

According to sizes:

Small lab and research fermenter – **1-50L**

Pilot plant fermenter - **50-1000L**

Large size industrial production scale fermenter- **more than 1000l**


Fermenter
Nature of media employed in fermentation/Substrate used
Suspended/submerged growth fermenter (SmF)
Solid surface fermenter/solid support system fermenter (SSF)
Type of Agitation
Mechanical stirrer/ stirred tank fermenter (STF)
Forced convection fermenter
Pneumatic fermenter
Tray fermenter
Packed / Bed column fermenter
1. Batch fermenter
2. Continous fermenter
1. Air lift fermenter
2. Bubble column fermenter
1. Fluidized bed fermenter

I. Submerged fermenters- in liquid media

In this type of fermenters, the cells are freely suspended in the culture medium, to grow and formation of product.

The agitation in media provides movements for the mixing of air / gases, allow better contact between cultured cells and substrate.

1. **Mechanical stirrer/ stirred tank fermenter (STF)**
2. **Forced convection fermenter**
3. **Pneumatic fermenter**

The common substrates used in submerged fermentation system are soluble sugars, vegetable juices, and sewage water

II. Supported growth fermenters---process occuring in the absence of water

These are also called surface culture fermenter system. In this type of fermenter, vegetative microbial cells are cultured on a support or surface, inside the culture media. The cells are not freely suspended in media.

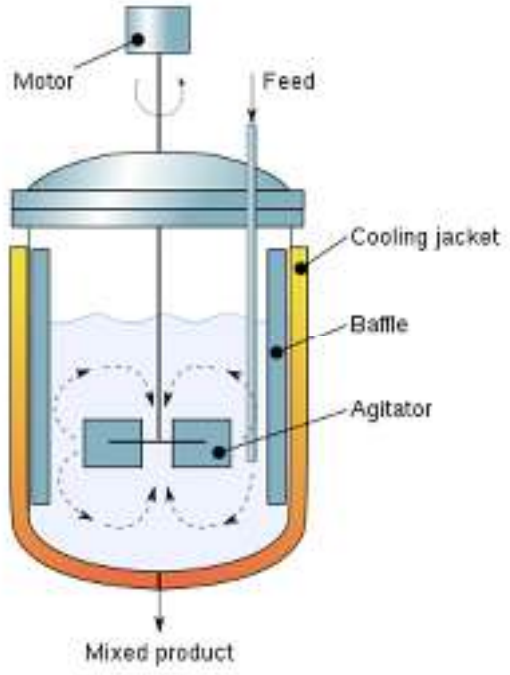
The main advantage of this is, better aeration, high product yield and easier product isolation or easy downstream process

1. **Tray fermenter**
2. **Packed / Bed column fermenter**

microbial cells utilized solid nutrient-rich waste materials such as grains, Bran, Bagasse, and paper pulp.

1. Mechanical stirrer/ stirred tank fermenter (STF)

They follow the structure of a basic fermenters, as they contains all basic parts of a typical fermenter, and a mechanical stirrer in centre to agitate the media filled inside fermenter. This agitation is necessary for mixing of filled material, heat transfer, mass transfer, and appropriate mixing if gases in media.



These are upright cylindrical shaped fermenters, and the selected volume should be more than 30-50% of the volume liquid or the media, which is to be filled in it.

Because the upper head space is required for gases and foam which is produced during fermentation.

Advantages : There are many advantages of STRs over other types. These include the efficient gas transfer to growing cells, good mixing of the contents and flexible operating conditions, besides the commercial availability of the bioreactors.

2. Forced Convection Fermenter

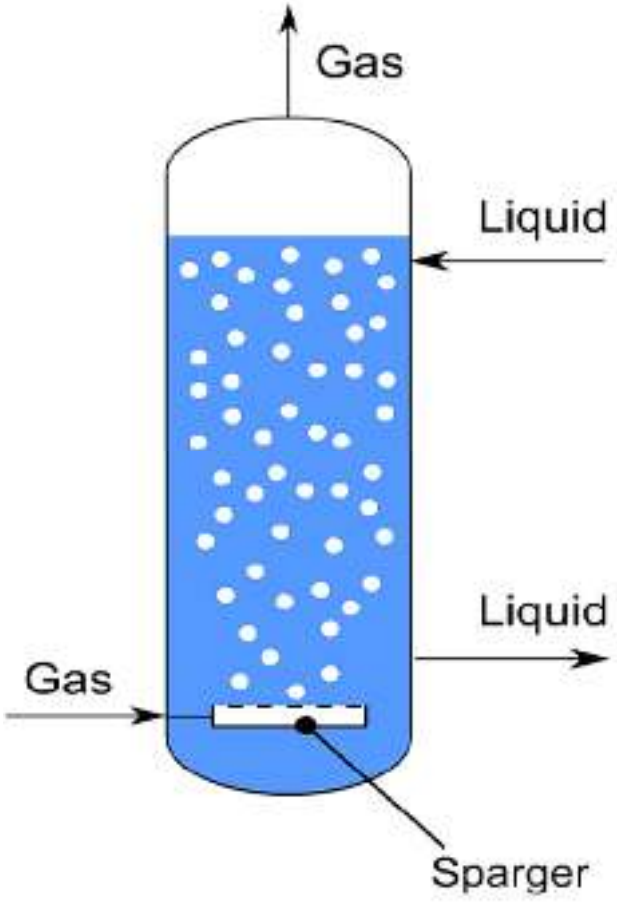
In these types of fermenters, the agitation is achieved through the movement of liquid and gas, by using pump. No mechanical impellers are employed for liquid movement.

They are two types: **A. Bubble Column Fermenter**
B. Air Lift Fermenter

A. Bubble Column fermenter or Sparged gas fermenter

These are simple types of convection fermenters. The height is three times of the diameter. The gas, usually air is sparged or introduce through the spargers, fitted at the bottom of the fermenter.

This gas / air moves upward and creates agitation in the medium. The jets of sparger are evenly distributed all over bottom to create uniform agitation throughout the media. These fermenters has no mechanical impellers, so they need less energy.



B. Air Lift Fermenter

The height is around 10 times than the diameter of these fermenter vessels. The movement of media is achieved by change in its density.

The gas is introduced at a specific area called riser. The presence of gas in the form of bubbles, reduces the density of liquid media, and this less density media moves upward.

At the top, the gas is gets separated from liquid, and due to this, liquid gets higher density, and moved downward. This creates a cyclic movement in liquid, cause agitation.

Airlift fermenter

- 1)Concentric draft tube airlift fermenter

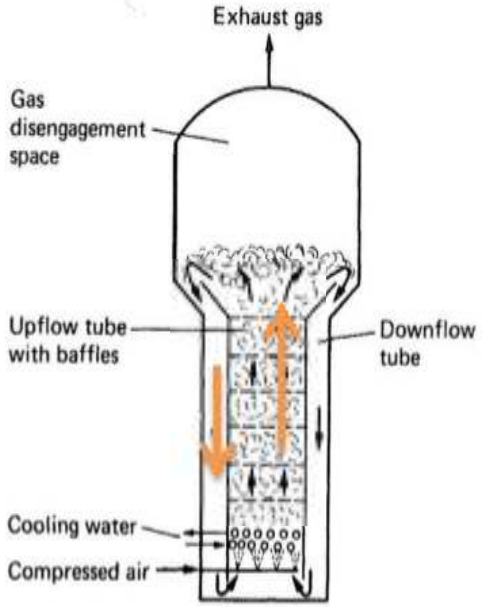


FIG. 7.47b. Air-lift fermenter with inner loop (Smith, 1980).

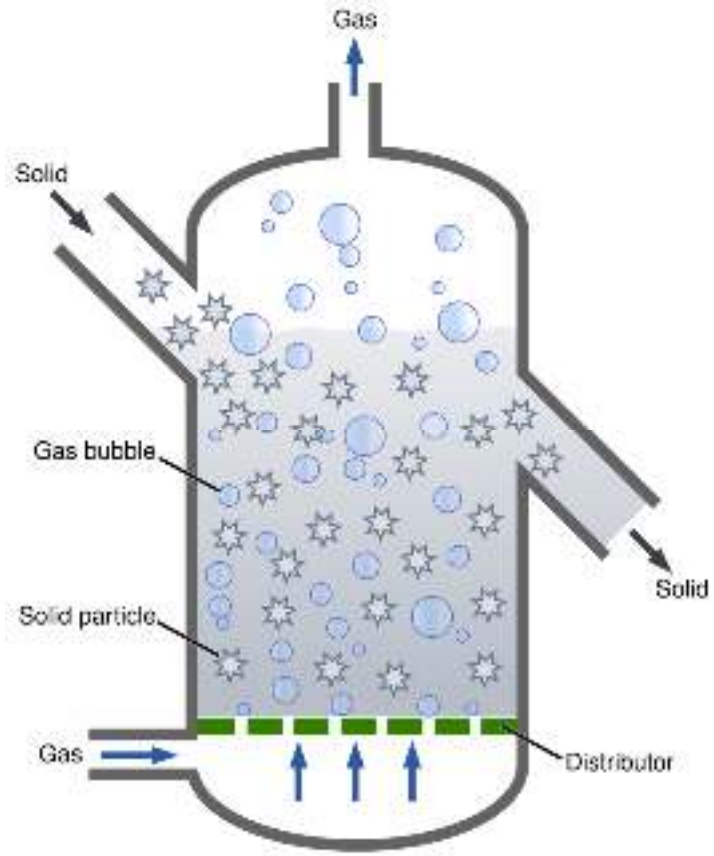
3. Pneumatic Fermenter: -

Fluidized bed fermenter

These are similar to column reactors, but upper part is more wider, to minimize the liquid flow speed. The microbial cells are grown on any spherical support like glass balls or any porous material, are suspended in liquid media.

From the bottom, the air gas introduce in such a way that it makes the balls to float inside liquid medium. The flow of gas and the density of support material is adjusted in such a way that the balls are not thrown outside. The liquid, and the gas pressure not such low that the balls are settled down at the bottom of the reactor.

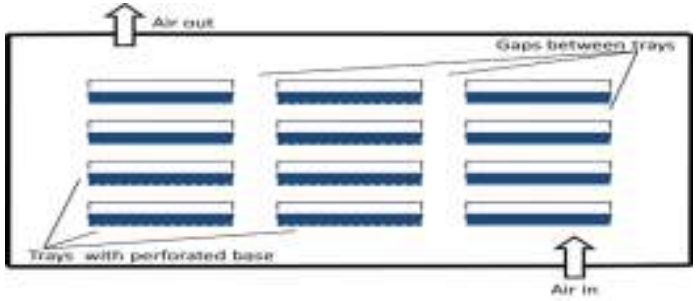
This type of reactor provides better contact of gas with the microbial cells.

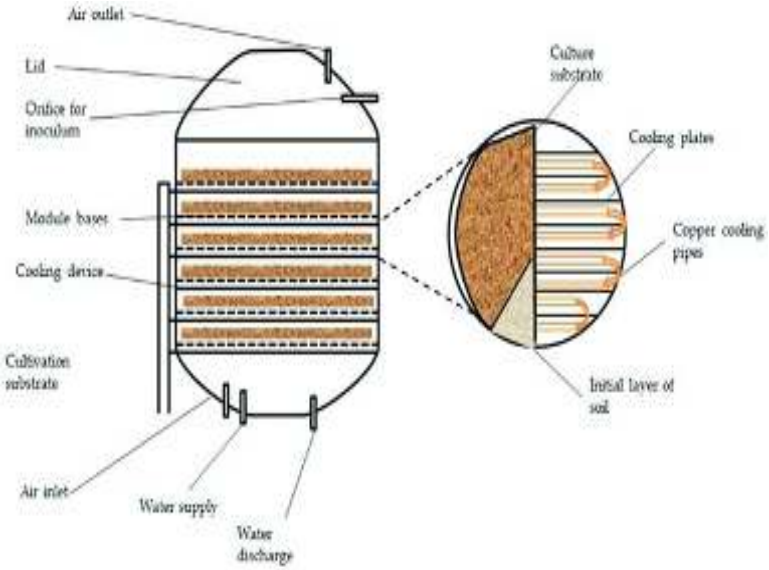


II. Supported growth fermenters

1. Tray fermenter:

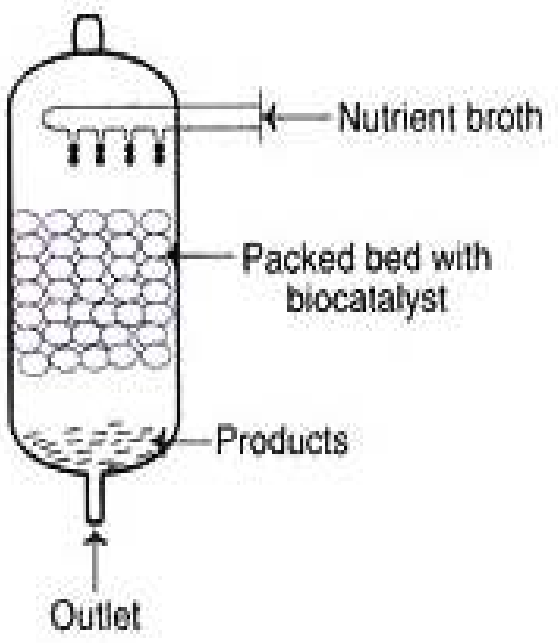
Tray fermenters are the simplest of all types of fermenters and may be wooden, metallic (aluminum or iron). If iron is used, it should be painted to avoid corrosion. The bottom is perforated in such a way that it holds substrate and allows aeration of the under surface of the substrate. Usually, trays are arranged one above the other with a suitable gap between trays. The fermentation is carried out in a chamber where a controlled humid atmosphere is created. The temperature of the fermenting substrate is controlled by circulating warm or cool air as necessary.





2. Packed / Bed column fermenter

A bed of solid particles, with biocatalysts on or within the matrix of solids, packed in a column constitutes a packed bed bioreactor. The solids used may be porous or non-porous gels, and they may be compressible or rigid in nature. A nutrient broth flows continuously over the immobilised biocatalyst. The products obtained in the packed bed bioreactor are released into the fluid and removed. While the flow of the fluid can be upward or downward, down flow under gravity is preferred.

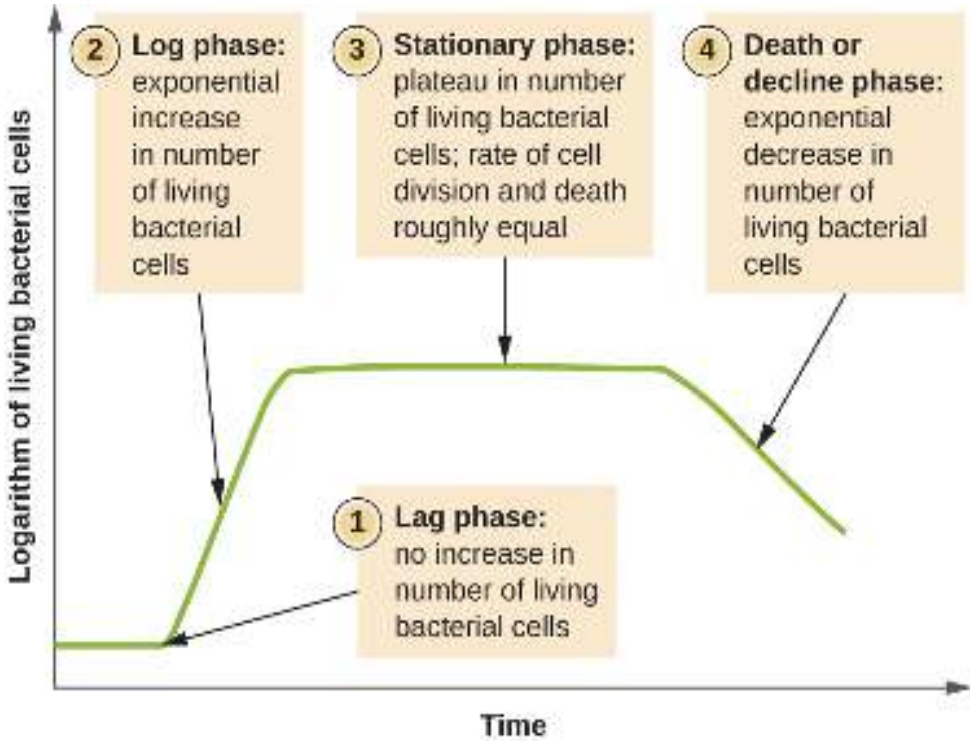


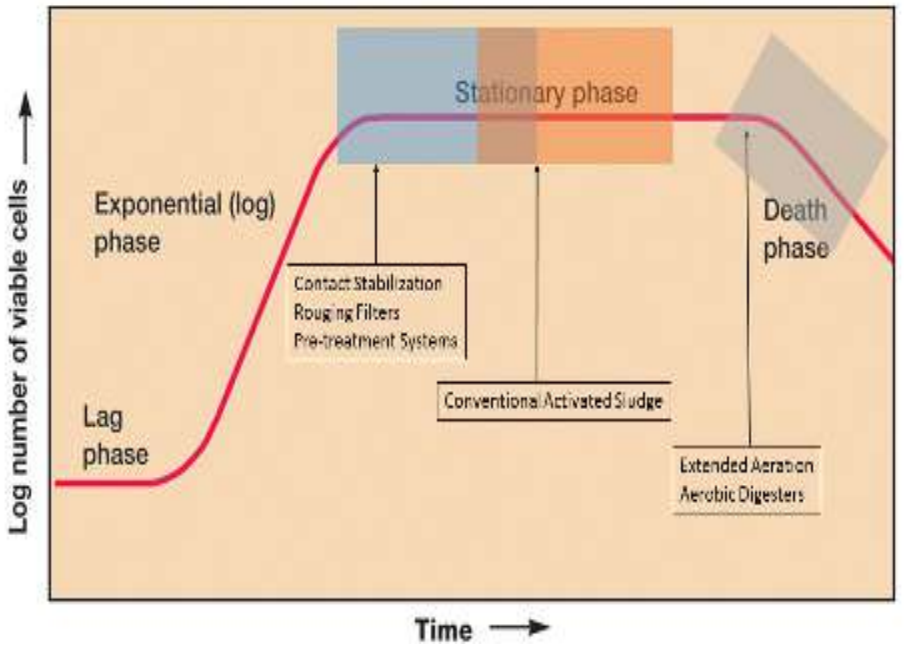
The concentration of the nutrients (and therefore the products formed) can be increased by increasing the flow rate of the nutrient broth. Because of poor mixing, it is rather difficult to control the pH of packed bed bioreactors by the addition of acid or alkali. However, these bioreactors are preferred for bioprocessing technology involving product-inhibited reactions. The packed bed bioreactors do not allow accumulation of the products to any significant extent.

The packed bed reactors are widely used with immobilized cells. Several modifications such as tapered beds to reduce the pressure drop across the length of the reactor, inclined bed, horizontal bed, rotary horizontal reactors have been tried with limited success.

FERMENTATION TYPES

Microbial - Growth Curve



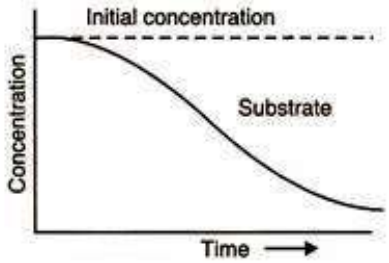


1. Batch Fermentation
2. Continuous Fermentation
3. Fed Batch Fermentation

1. Batch Fermentation

A batch fermentation is a closed culture system, because initial and limited amount of sterilized nutrient medium is introduced into the fermenter. The medium is inoculated with a suitable microorganism and incubated for a definite period for fermentation to proceed under optimal physiological conditions. Oxygen in the form of air, an antifoam agent and acid or base, to control the pH, are being added during the course of fermentation process.

During the course of incubation, the cells of the microorganism undergo multiplication and pass through different phases of growth and metabolism due to which there will be change in the composition of culture medium, the biomass and metabolites. The fermentation is run for a definite period or until the nutrients are exhausted. The culture broth is harvested and the product is separated.



The used medium along with cells of microorganism and the product is drawn out from the fermenter. When the desired product is formed in optimum quantities, the product is separated from the microorganism and purified later on

Lag phase -adapt to their surroundings

Exponential growth - grow in numbers

Stationary phase -stop growing

Death phase

Merits:

(a) The possibility of contamination and mutation is very less.

(b) Simplicity of operation and reduced risk of contamination.

Demerits:

(a) For every fermentation process, the fermenter and other equipment are to be cleaned and sterilized.

(b) Only fraction of each batch fermentation cycle is productive.

(c) It is useful in fermentation with high yield per unit substratum and cultures that can tolerate initial high substrate concentration.

(d) It can be run in repeated mode with small portion of the previous batch left in the fermenter for inoculum.

(e) Use of fermenter is increased by eliminating turn round time or down time.

(f) Running costs are greater for preparing and maintaining stock cultures.

(g) Increased, frequency of sterilization may also cause greater stress on instrumentation and probes.

(h) Fresh sterilized medium and pure culture are to be made for every fermentation process.

(i) Yield of the desired product may also vary.

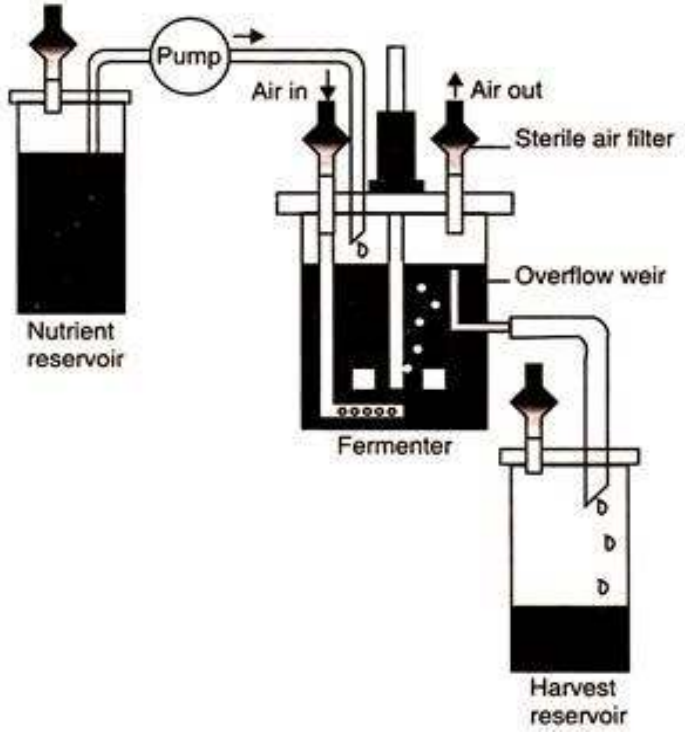
(j) There will be a non-productive period of shutdown between one batch productive fermentation to the other,

(k) More personal are required.

2. Continuous Fermentation:

It is a closed system of fermentation, run for indefinite period. In this method, fresh nutrient medium is added continuously or intermittently to the fermenter and equivalent amount of used medium with microorganisms is withdrawn continuously or intermittently for the recovery of cells or fermentation products.

As a result, volume of the medium and concentration of nutrients at optimum level are being maintained. This has been operated in an automatic manner. The continuous fermenter has its maximum use that take long time to reach high productivity, reduces down time and lowers the operating costs.



In continuous mode, starting medium and inoculum are added to the fermenter. After the culture is grown the fermenter is fed with nutrients and broth is withdrawn at the same rate maintaining a constant volume of broth in the fermenter. In continuous mode with cell cycle, the cell mass is returned to the fermenter using micro filtrations with bacteria or screens with fungal mycelium.

Merits:

1. The fermenter is continuously used with little or no shutdown time.
2. Only little quantity of initial inoculum is needed and there is no need of additional inoculum.
3. It facilitates maximum and continuous production of the desired product.
4. There is optimum utilization of even slow utilizable substances like hydrocarbons.

Demerits:

1. Possibility of contamination and mutation because of prolonged incubation and continuous fermentation, are more.
2. Possibility of wastage of nutrient medium because of continuous withdrawal for product isolation.
3. The process becomes more complex and difficult to accomplish when the desired products are antibiotics rather than a microbial cells.
4. Lack of knowledge of dynamic aspects of growth and synthesis of product by microorganism used in fermentation.

Applications:

Continuous culture fermentation has been used for the production of single cell protein, antibiotics, organic solvents, starter cultures.

3. Fed Batch Fermentation:

It is a modification to the batch fermentation. In this process substrate is added periodically in instalments as the fermentation progresses, due to which the substratum is always at an optimal concentration. This is essential as some secondary metabolites are subjected to catabolite repression by high concentration of either glucose, or other carbohydrate or nitrogen compounds present in the medium.

For this reason, the critical elements of the nutrient medium are added in low amount in the beginning of the fermentation and these substrates continue to be added in small doses during the production phase. This method is generally employed for the production of substances such as penicillin. Yoshida (1973) introduced this term for the first time for feeding the substrates to the medium as the nutrients are exhausted, so as to maintain the nutrients at an optimum level.

ANTIGEN-ANTIBODY INTERACTIONS

By

Dr Arunima Karkun

ANTIGEN-ANTIBODY INTERACTIONS

SYNOPSIS

- INTRODUTION
- DEFINITION
- FEATURES
- TYPES
- MECHANISM
- APPLICATION
- CONCLUTION
- REFRENCES

ANTIGEN-ANTIBODY INTERACTIONS

INTRODUCTION

- **Antigen-antibody reaction** is the basis of **humoral immunity** or antibody mediated **immune response.**
- The noncovalent interactions that form the basis of antigen -antibody (Ag-Ab) binding include **hydrogen bonds**, **ionicbonds**, **hydrophobic interactions,** and **van der Waals interactions.**
- Antigen-antibody interactions depend on four types of noncovalent interactions: hydrogen bonds, ionic bonds, hydrophobic interactions, and vander Waals interactions.

ANTIGEN-ANTIBODY INTERACTIONS

INTRODUCTION

ANTIGEN – ANTIBODY REACTION -

- An antibody combines specifically with the corresponding antigen or hapten in a manner which is very similar to the binding of a enzyme to it is substrate and involving hydrophobic and ionic interaction.

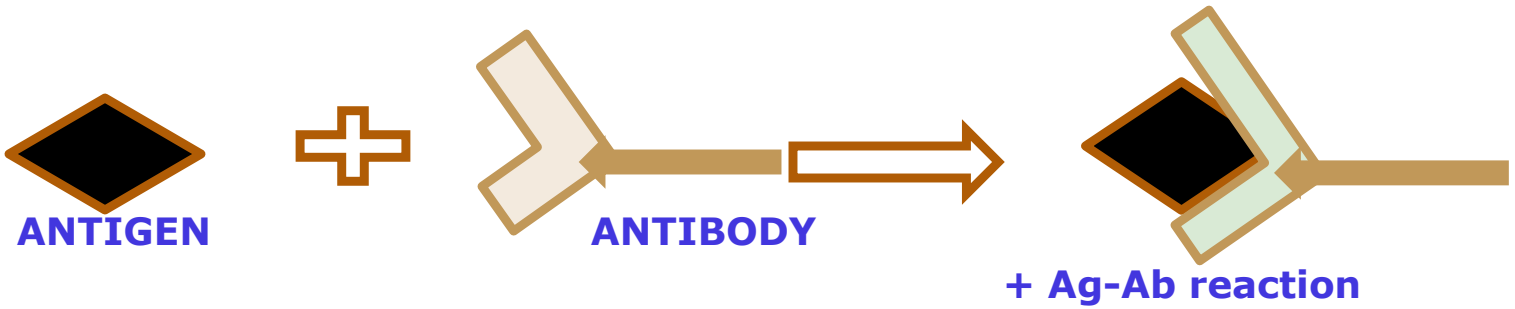


FIG :- 1 ANTIGEN – ANTIBODY REACTION

ANTIGEN-ANTIBODY INTERACTIONS

DEFINITION

- "The interaction between antigen & antibody is called antigen– antibody reaction. It is abbreviated as **Ag-Ab reaction.**"

ANTIGEN-ANTIBODY INTERACTIONS

FEATURES

1. IMMUNE COMPLEX
2. SPECIFICITY OF Ag- Ab REACTION
3. BINDING SITES OF ANTIGEN-ANTIBODY
4. BINDING FORCES OF ANTIGEN & ANTIBODY
5 . AVIDITY
6. BONUS EFFECT
7. CROSS -REACTION

ANTIGEN-ANTIBODY INTERACTIONS

IMMUNE COMPLEX

1. IMMUNE COMPLEX

- When antigen & antibody are brought together , the antibody binds with the antigen to form a complex molecules called **immune complex** or Ag- Ab complex**.**
- **Ag + Ab ⟶ Ag – Ab COMPLEX**

FIG :- 2 ANTIGEN – ANTIBODY REACTION

ANTIGEN-ANTIBODY INTERACTIONS

BINDING SITES

2. BINDING SITES OF ANTIGEN AND ANTIBODY

- In antigen-antibody reaction the antibody attached with the antigen.
- The part of the antigen which combines with the antibody is called **epitope** or antigenic determinates.
- An antigen may contain 10 to 50 antigenic determinants.
- Some time it may go up to 200.
- The part of the antibody which combines with the antigen is called **paratope** or antigen binding site.
- Most of the antibodies are bivalent having two binding sites.
- But the antibody IgM is multivalent having 5 to 10 binding sites.

ANTIGEN-ANTIBODY INTERACTIONS

BINDING SITES

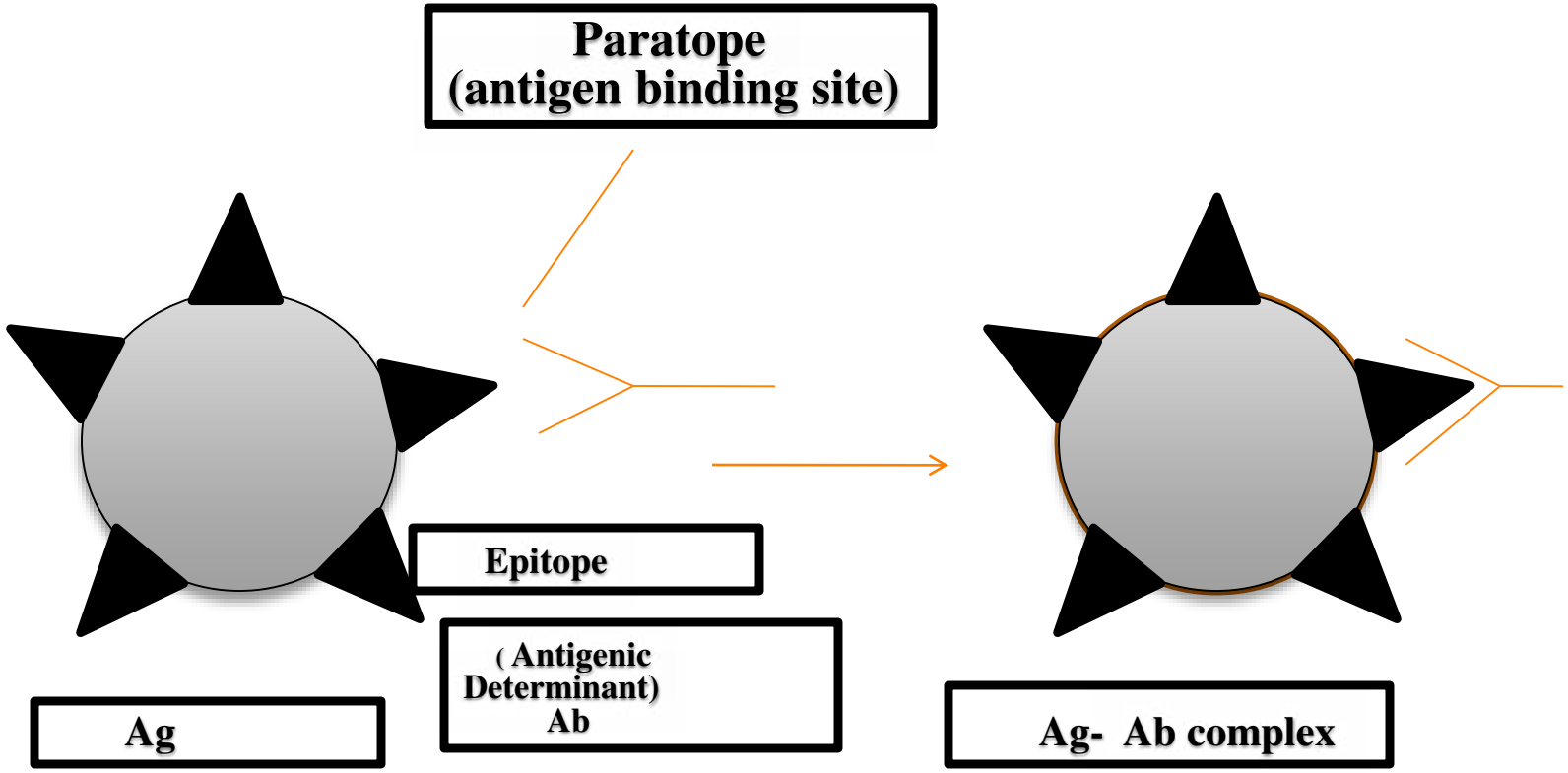


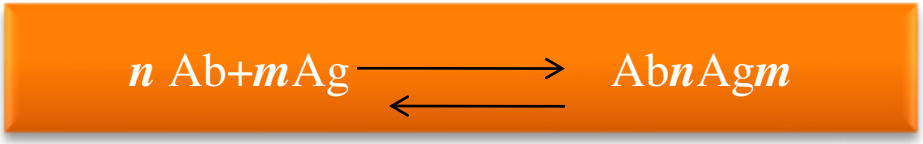
FIG:- 3 ANTIGEN AND ANTIBODY TO SHOW EPITOPE & PARATOPE

ANTIGEN-ANTIBODY INTERACTIONS

AVIDITY

3. AVIDITY

- Avidity refer to be capacity of an antiserum containing to combine with the whole antigen that stimulated the production of antibodies.



- Where n Ab=number of antibodies
- mAg=Antigentic determinants

- A multivalent antigen has many type of antigenic determinant stimulates the production of a particular antibody .

ANTIGEN-ANTIBODY INTERACTIONS

AVIDITY

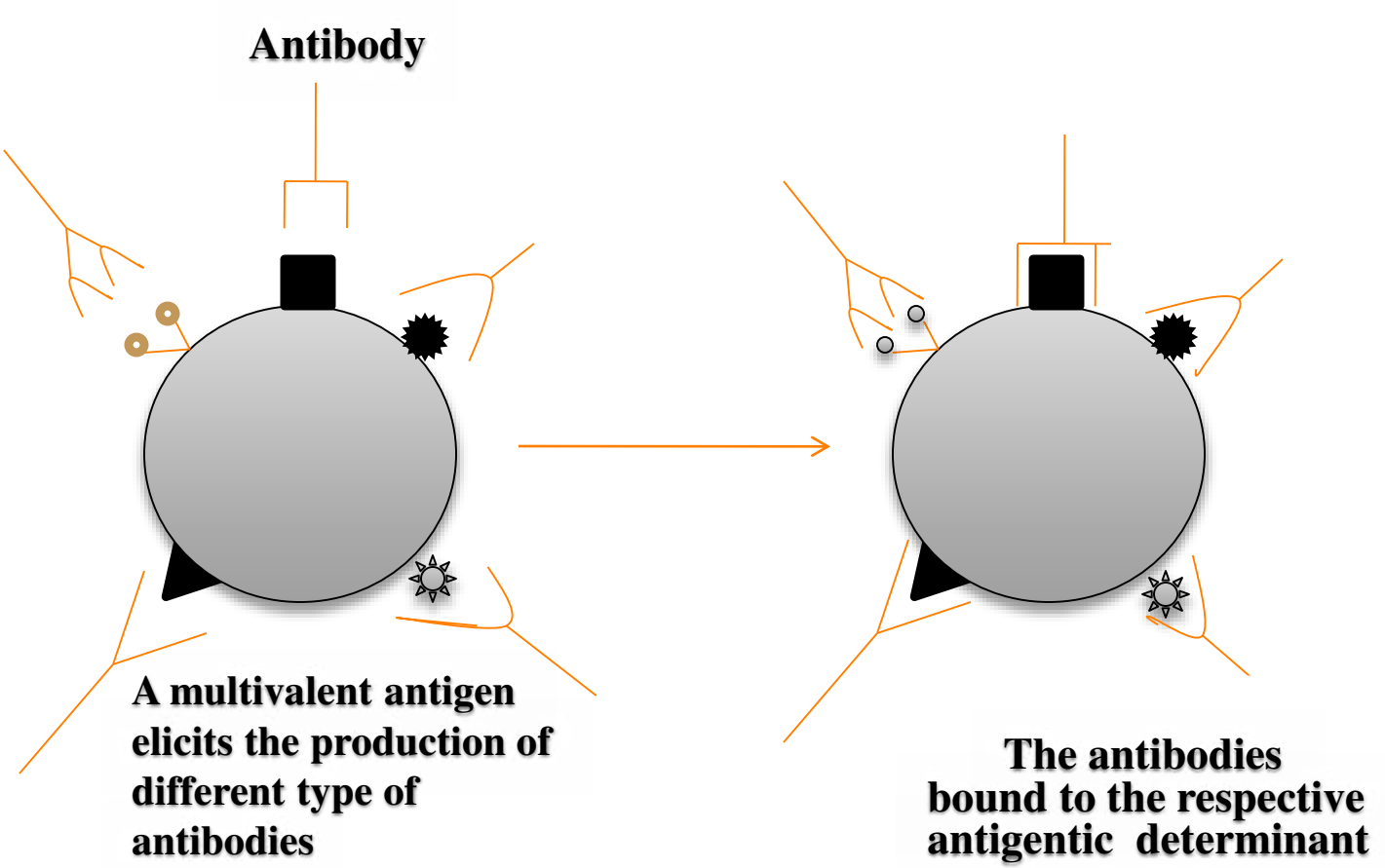


FIG :- 4 AVIDITY.

CROSS REACTION

4. CROSS REACTION

- An antiserum raised against a given antigen may sometimes react with another closely related antigen.
- This reaction is called cross reaction. & the antigen which produce the cross reaction is called **cross reactive antigen.**
- The **cross reaction** is due to the presence one or more identical antigenic determinants on the related antigen.

ANTIGEN-ANTIBODY INTERACTIONS

CROSS REACTION

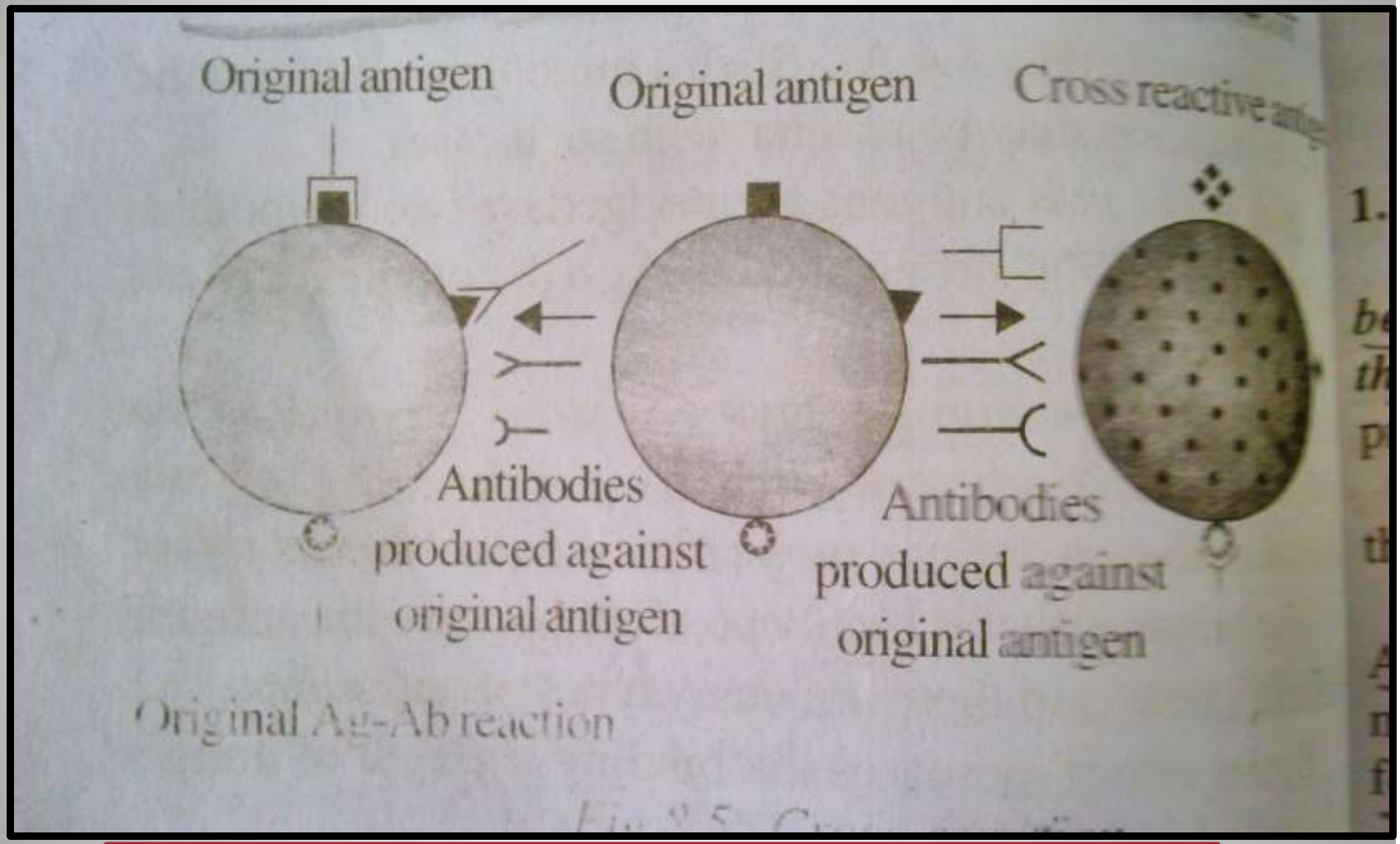


FIG: - 5 CROSS-REACTION

ANTIGEN-ANTIBODY INTERACTIONS

DETECTION

1. PRECIPITATION

- ✓ Precipitation refers to an *antigen – antibody reaction* between a soluble antigens &its antibody resulting in the formation of insoluble Precipitate. the antibody causing Precipitation is called **Precipitation** .

❑ MECHANISM

- ✓ Precipitation is due to the formation of *antigen – antibody complex.*
- ✓ The *antigen* is multivalent & the antibody is bivalent .
- ✓ As each antibody is a bivalent molecules ,it can bridge two multivalent antigen molecules.
- ✓ This bridge leads to the formation of a lattice which forms the Precipitate.
- ✓ When antigen & antibody are in optimal concentration, the Precipitation is complete & a large lattice is formed.

ANTIGEN-ANTIBODY INTERACTIONS

PRECIPITATION

PRECIPITATION TEST –

- ✓ precipitin test is a test of antigen – antibody reaction.
- ✓ precipitin reaction can be carried out by a classical experiment .
- ✓ A set of 5 or more reaction tubes are arranged serially & are marked as A, B , C, D, E .
- ✓ A constant volume of antiserum is added to each tubes.
- ✓ the antigen is added in increasing volume form tube A to E.
- ✓ antigen & antibody react together resulting in precipitation.
- ✓ The amount of precipitate formed is determined by the proportion of antigen & antibody.
- ✓ when the amount of precipitate formed in different tubes is plotted on a graph paper a curve is obtained. this curve is called **precipitin curve.**

ANTIGEN-ANTIBODY INTERACTIONS

PRECIPITATION

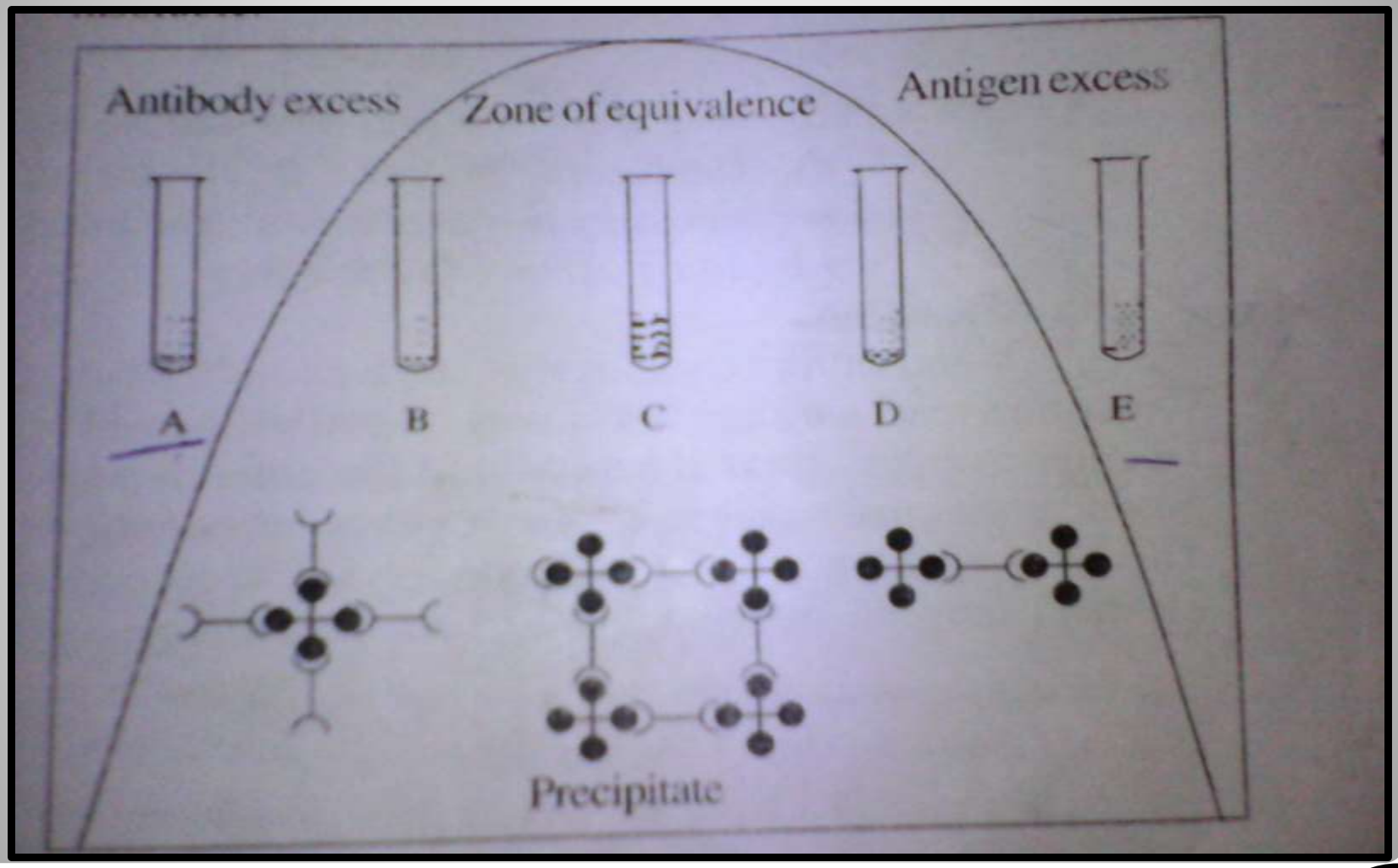


FIG :- 6 PRECIPITATION TEST

ANTIGEN-ANTIBODY INTERACTIONS

PRECIPITATION

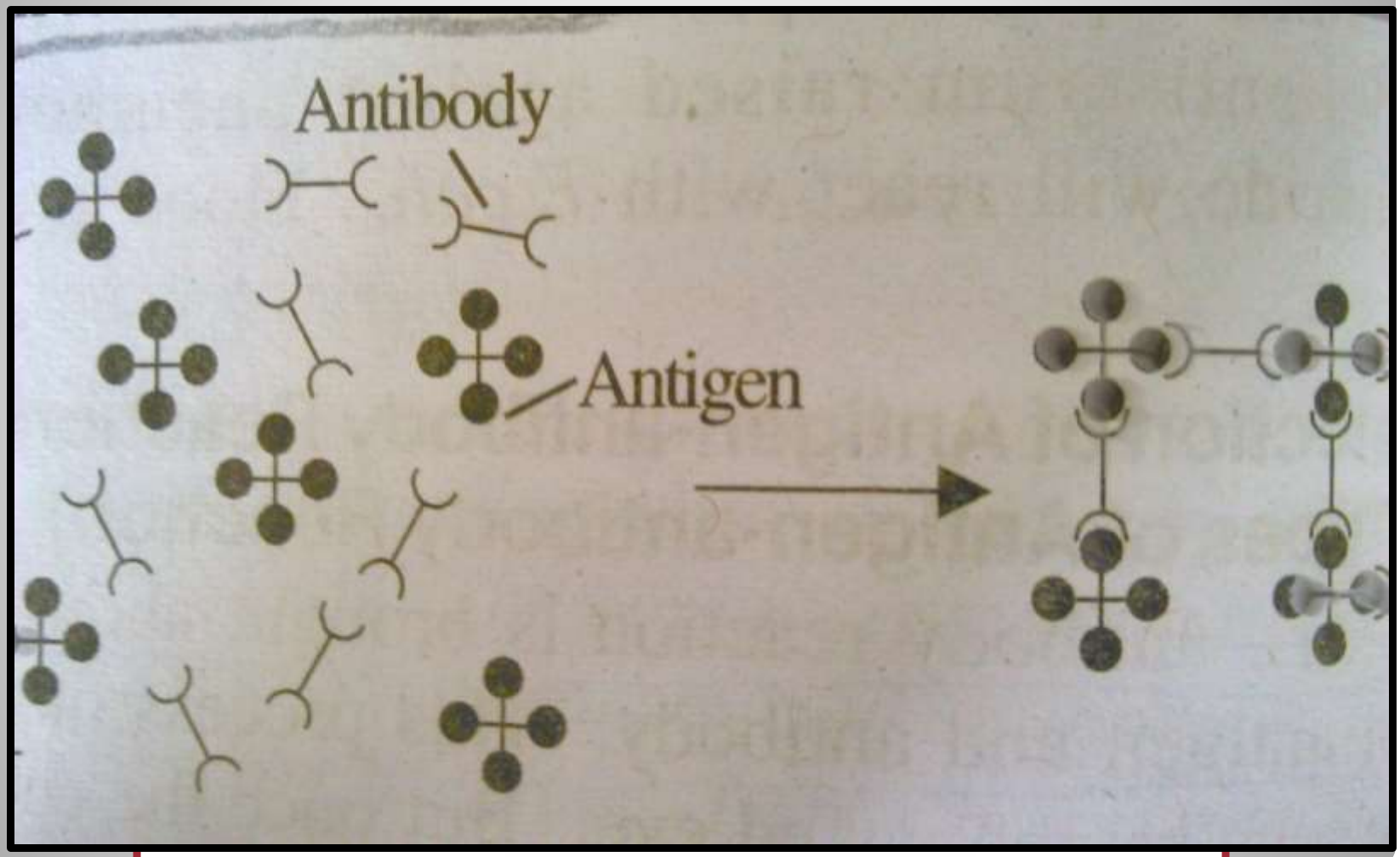


FIG:- 7 PRECIPITATION

ANTIGEN-ANTIBODY INTERACTIONS

PRECIPITATION

The precipitin curve shows 3 zones, namely :-

- Zone of antibody excess
- Zone of equivalence
- Zone of antigen excess

APPLICTION

- Single immunodiffusion
- Dabble immunodiffusion
- Radio Immuno assay(RIA)
- Immuno electrophoresis
- Rockets immunodiffusion

ANTIGEN-ANTIBODY INTERACTIONS

R I A

APPLICATION

RADIOIMMUNOASSAY (RIA)-

- **Radioimmunoassay** is one of the most important techniques in the clinical biochemical fields for the quantitative analysis of hormones, and drugs .
- It combines the specificity of the immune reaction with the sensitivity of the radioisotope techniques.
- The most commonly used labels are **radioisotope** and **enzymes.**
- A variety of tests have been devised for the measurement of antigen and antibodies using such labeled reactants.

ANTIGEN-ANTIBODY INTERACTIONS

R I A

- **APPLICATION**

- This techniques is also useful in diagnosing insulinomas, sex hormone sensitive tumors etc. and this facilitates proper treatment of the disease.

- Estimation of peptides steroids hormone, vitamins, drugs, antibodies nucleic acids, structural proteins hormone receptor proteins.

- Radioimmunoassay has tremendous application in the diagnosis of hormonal disorders, cancers and therapeutic monitoring of drugs besides being useful in biomedical research.

- The most sensitive techniques for detecting antigen or antibody is radioimmunoassay (RIA).

ANTIGEN-ANTIBODY INTERACTIONS

AGGLUTINATION

2. AGGLUTINATION

- Agglutination is an antigen –antibody reaction where the antibody of serum causes the cellular antigen to adhere to one another to form clumps.
- It is the clumping of a particular antigen and its antibody.
- The antibody that cause agglutination are called agglutinins and particulate antigens aggregated are called agglutinogens.
- The particulate antigen include bacterial ,viruses ,RBC ,platelets lymphocytes ,etc.
- When red blood called are agglutinated ,the reaction is called **Heamagglutination .**
- When bacterial cells are agglutinated ,the agglutination is called **Bacterial Agglutination.**

ANTIGEN-ANTIBODY INTERACTIONS

AGGLUTINATION

MECHANISM OF AGGLUTINATION

- Agglutinations is brought about by the linking of antigen and antibodies.
- As most of the antibodies are bivalent ,an antibody can link two adjacent antigens.
- The IgM antibody is multivalent and it contains 5 0r 10 combining sites.
- Hence IgM antibody has the capacity to make clumps more effectively with a lesser number of molecules then that of IgM antibody molecule.
- The univalent antibodies (antibodies with a single combining site) cannot form clump or lattice and hence agglutination will not occur.

ANTIGEN-ANTIBODY INTERACTIONS

AGGLUTINATION

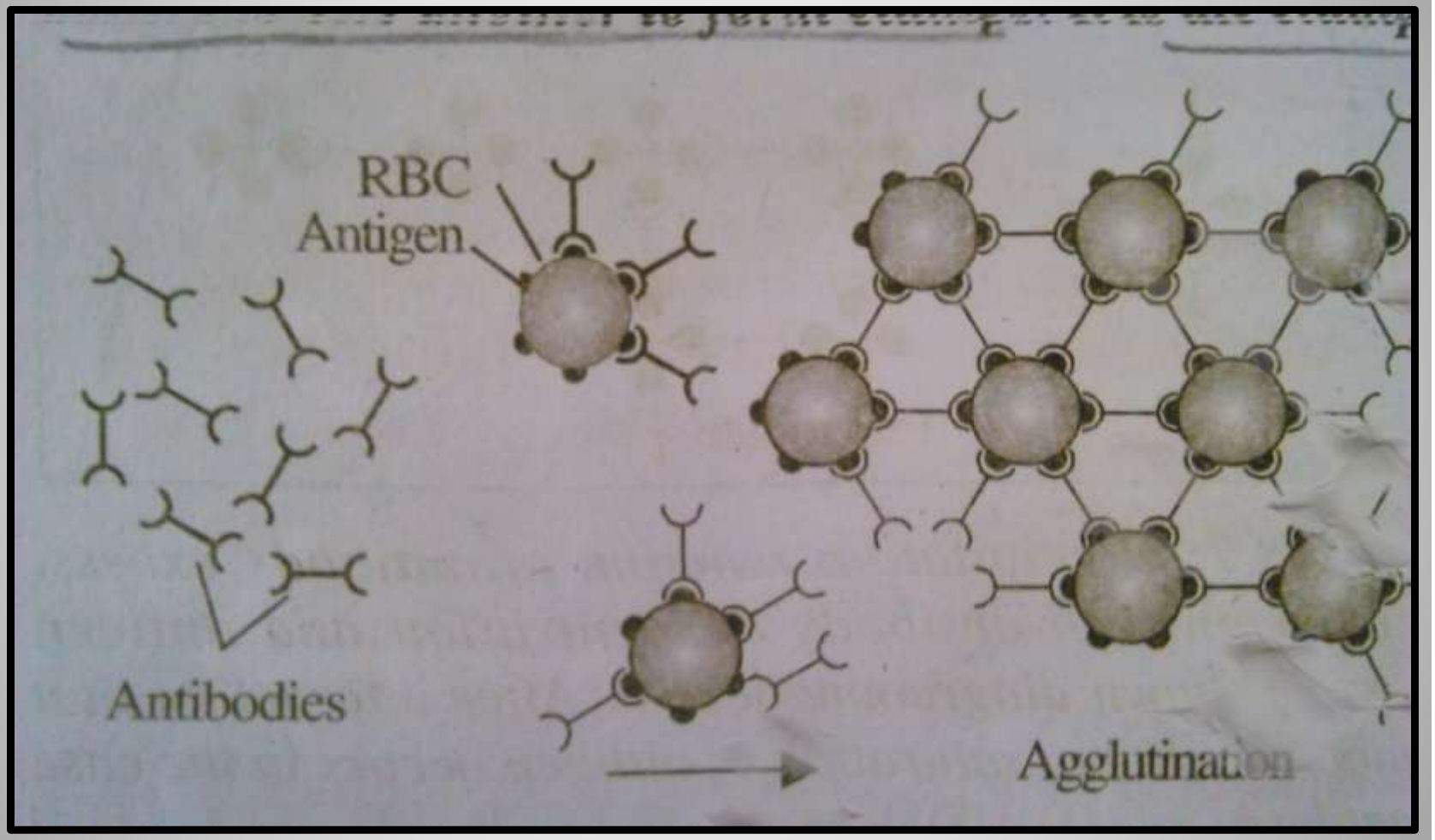


Fig :- 8 AGGLUTINATION

ANTIGEN-ANTIBODY INTERACTIONS

AGGLUTINATION

AGGLUTINATION TEST:

Agglutination test refer to the examination of clump formation when particular antigen and its antibodies are combined.

- ABO blood group
- Rh blood group
- Widal test for typhoid
- Coomb's test for the identification of anti - Rh antibodies

ANTIGEN-ANTIBODY INTERACTIONS

AGGLUTINATION

❖ EXAMPLE

ABO BLOOD GROUP –

- The typing of blood , for ABO groups or Rh groups,involves agglutination reaction.
- For typing blood , a drop of the blood sample is mixed with a drop of antiserum A & another drop of the blood sample is mixed with a drop of antiserum B on a glass slide.
- If belongs to is clumped with antiserum A , the sample belongs to belongs to , if the sample is clumped with antiserum B , if the sample is clumped with both antiserum A & antiserum B , the blood sample belongs to belongs to group AB . If there is no agglutination the blood sample belongs to group O.

ANTIGEN-ANTIBODY INTERACTIONS

AGGLUTINATION

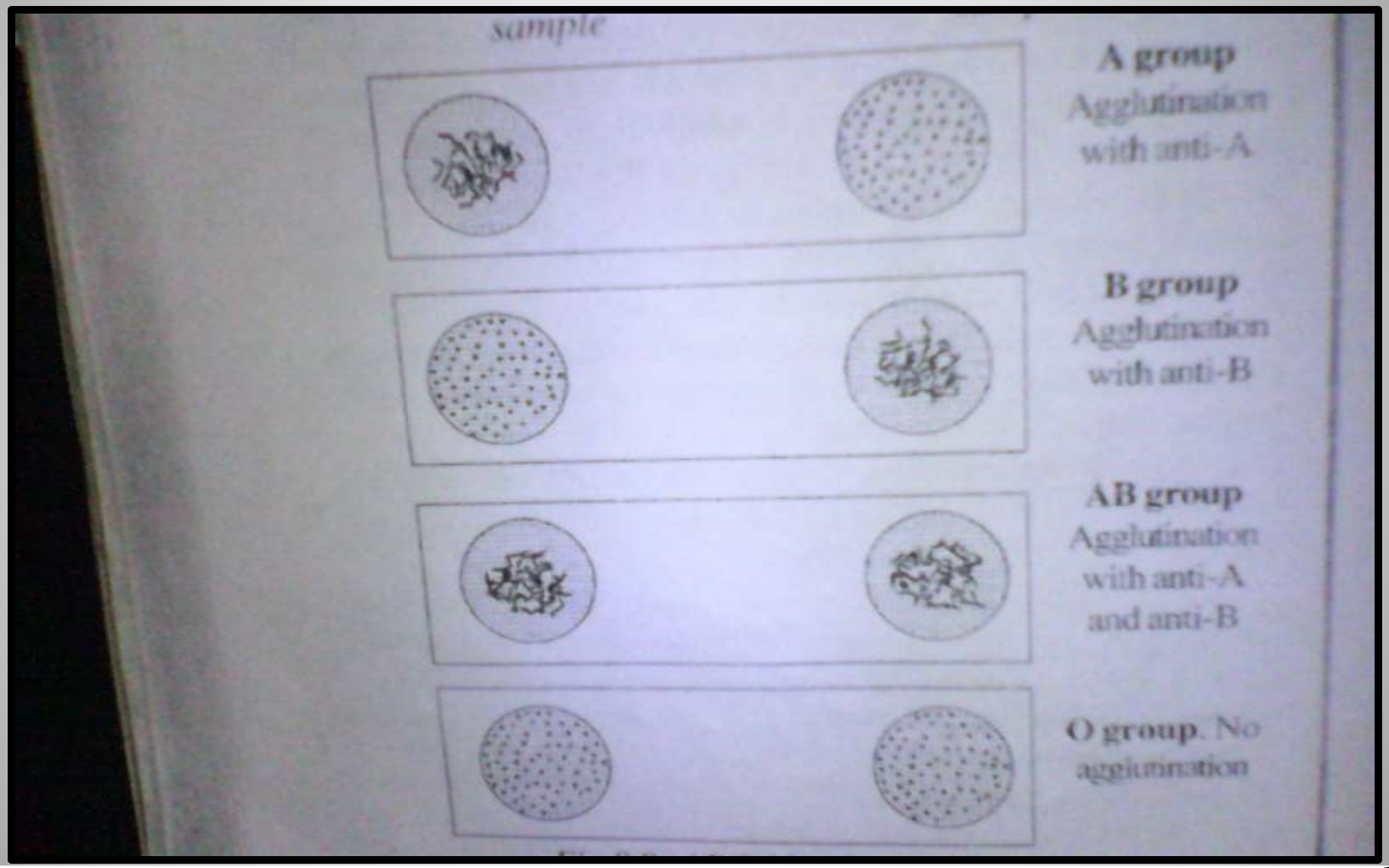


FIG :- 9 AGGLUTINATION

ANTIGEN-ANTIBODY INTERACTIONS

E L I S A

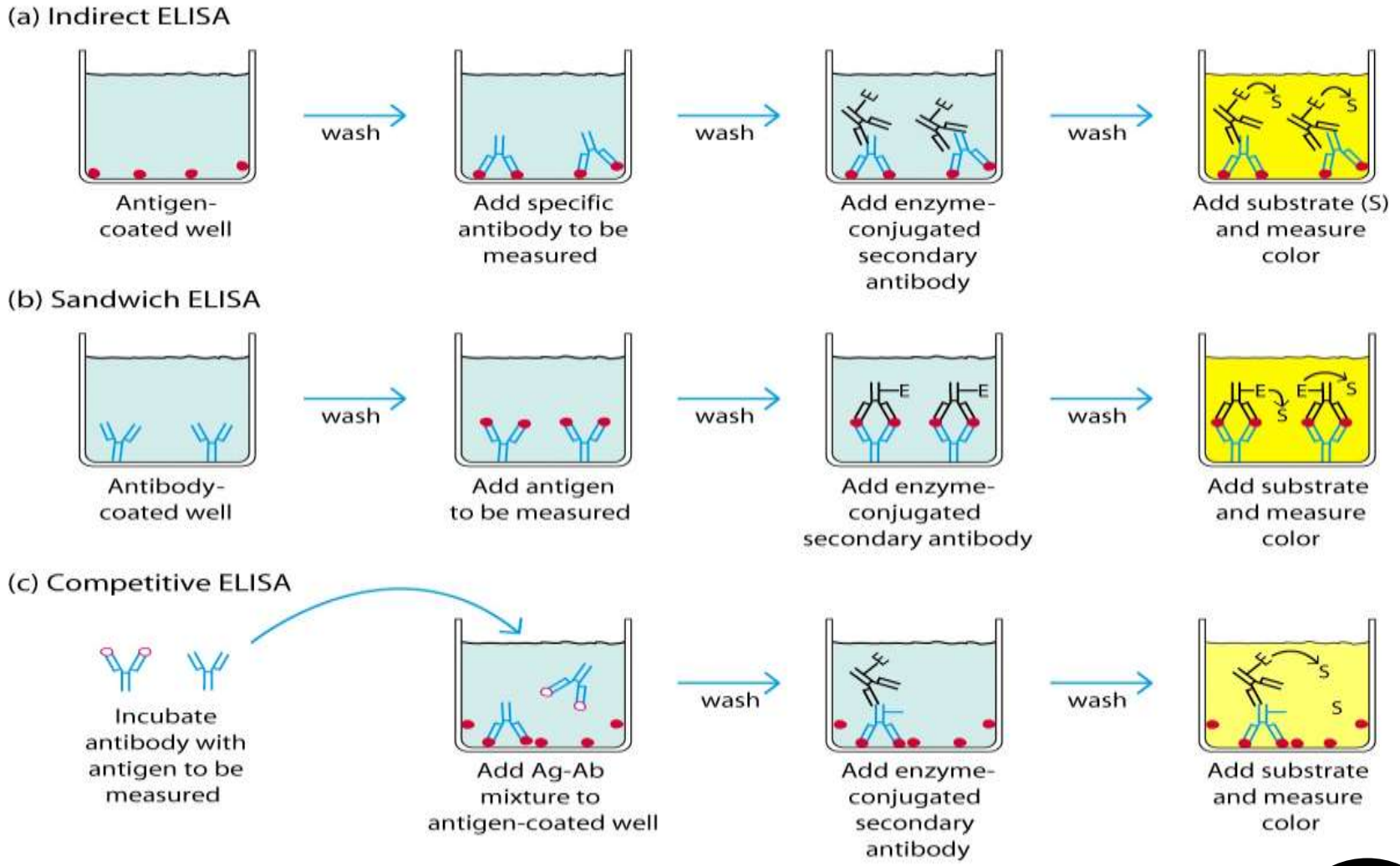
- **ELISA** also known as an **enzyme linked immunosorbent assay** is a biochemical Techniques used mainly in immunology to detected the Presence Of an **antibody** or an **antigen** in a sample.

- **Enzyme-linked immunosorbent assay, commonly known as ELISA or EIA**), is similar in principle to RIA but depends on an enzyme rather than **a radioactive label.**

- **ELISA** can also be used in toxicology as a rapid presumptive screen for certain classes of drug.

ANTIGEN-ANTIBODY INTERACTIONS

ELISA



ANTIGEN-ANTIBODY INTERACTIONS

ELISA

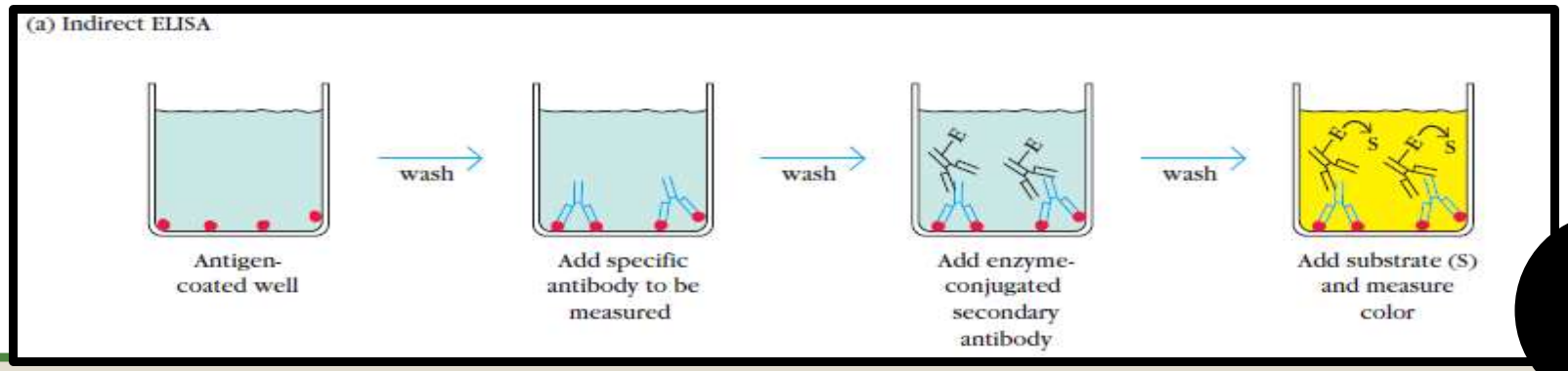
- **Enzyme Linked Immunosorbent Assay (ELISA)**
- Term Was Coined By ***Engvall*** and ***Pearlmann*** in **1971**
- **Different Type**
  - ✓ Indirect ELISA
  - ✓ Sandwich ELISA
  - ✓ Competitive ELISA
- **ELISA** used in the detection and quantization of several antigen as well as antibodies.
- Indirect **ELISA** method to detect the presence of serum antibody against HIV. The causative agent of AIDS.

ANTIGEN-ANTIBODY INTERACTIONS

INDIRECT ELISA

INDIRECT ELISA-

- Antibody can be detected or quantitatively determined with an indirect ELISA .
- Serum or some other sample containing primary antibody (Ab1) is added to an antigen-coated microtiter well .
- After any free Ab1 is washed away, the presence of antibody bound to the antigen is detected by adding an enzyme-conjugated secondary antibody (Ab2).
- Any free Ab2 then is washed away, and a substrate for the enzyme is added.
- The amount of colour reaction product that forms is measured by specialized spectrophotometric plate readers.

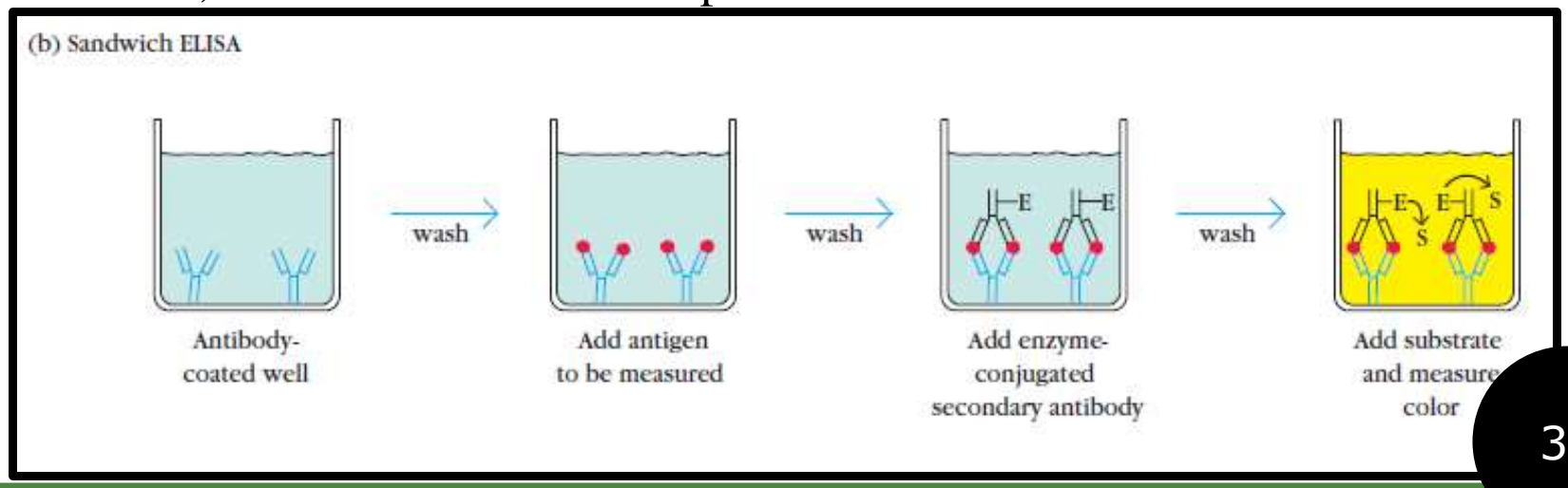


ANTIGEN-ANTIBODY INTERACTIONS

SANDWICH ELISA

❑SANDWICH ELISA-

- In this technique, the antibody (rather than the antigen) is immobilized on a microtiter well.
- A sample containing antigen is added and allowed to react with the immobilized antibody.
- After the well is washed, a second enzyme-linked antibody specific for a different epitope on the Antigen is added and allowed to react with the bound antigen.
- After any free second antibody is removed by washing, substrate is added, and the colour reaction product is measured.

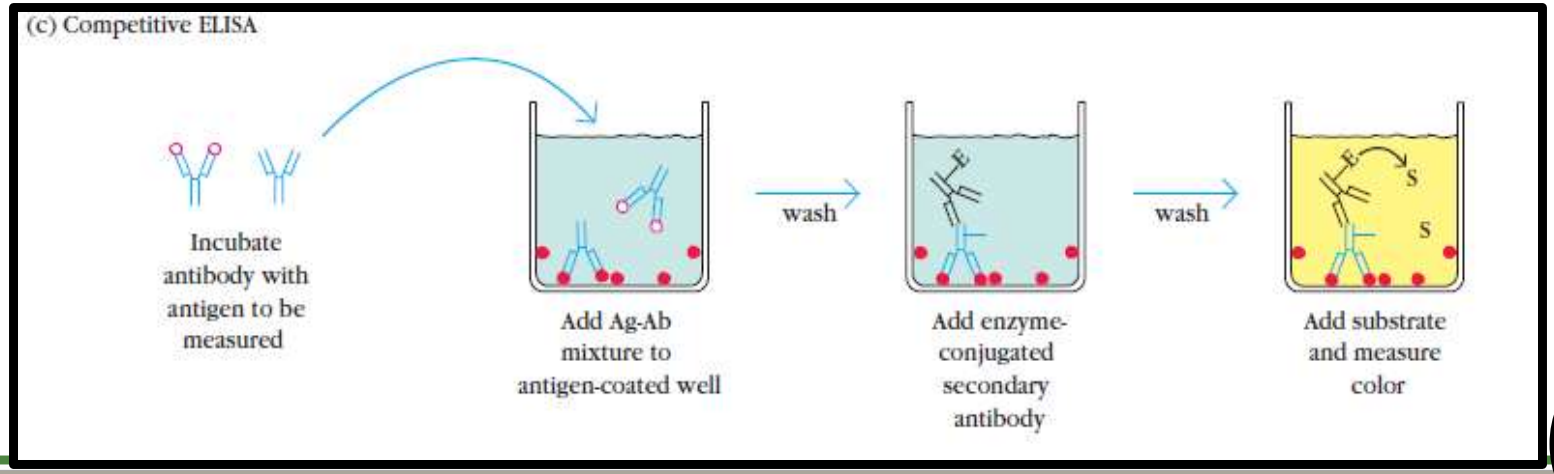


ANTIGEN-ANTIBODY INTERACTIONS

COMPETITIVE ELISA

COMPETITIVE ELISA

- In this technique, antibody is first incubated in solution with a sample containing antigen.
- The antigen-antibody mixture is then added to an antigen coated microtiter well.
- The more antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well.
- In the competitive assay, however, the higher the concentration of antigen.



ANTIGEN-ANTIBODY INTERACTIONS

CONCLUSION

- **Antigen-antibody reaction** is the basis of humoral immunity or antibody mediated immune response.
- The noncovalent interactions that form the basis of antigen -antibody (Ag-Ab) binding include **hydrogen bonds**, **ionicbonds**, **hydrophobic interactions,** and **van der Waals interactions.**
- **ELISA** also known as an enzyme linked immunosorbent assay is a biochemical Techniques used mainly in immunology to detected the Presence Of an **antibody** or an **antigen** in a sample.
- **Radioimmunoassay** is one of the most important techniques in the clinical biochemical fields for the quantitative analysis of hormones, and drugs .

ANTIGEN-ANTIBODY INTERACTIONS

REFRENCES

BOOK NAME	AUTHER NAME	EDITION
IMMUNOLOGY	PROF. DULSY FATIMA	1ST Edition
IMMUNOLOGY W.H.FREEMAN	JAINS KUBY	6th Edition
MICROBIOLOGY	ANANTHANARAYAN & PANIKER'S	8TH Edition-
INTERNET SOURCES &CLASSNOTES-	2013	


THANK YOU

UNIT III - IMMUNOLOGY

- ✓ Principles of Immunity,
- ✓ Humoral immunity, cell mediated immunity,
- ✓ Antigen – antibody reactions,
- ✓ Hypersensitivity and its applications.
- ✓ Active & passive immunizations vaccine preparation, standardization & storage of BCG, cholera, smallpox, polio, typhus, tetanus toxoid, immuno serum & diagnostic agents.

1. INTRODUCTION

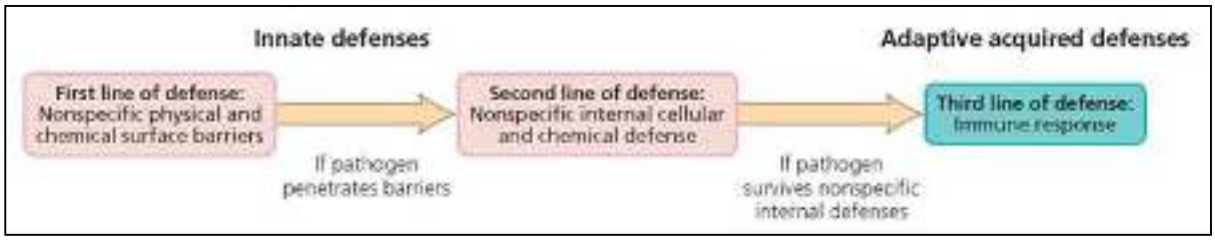
The body is protected from infectious agents and other harmful substances by a variety of cells and molecules that make up the immune system. Immunity is the ability of the human body to tolerate the presence of material indigenous to the body (self), and to eliminate foreign (non-self) material. Foreign substances such as viruses, bacteria, toxins, and parasites are surrounded by antigens that, when introduced into the body, are capable of inducing a response by the immune system. This discriminatory ability provides protection from infectious disease, since most agents or associated toxins are identified as foreign by the immune system.

Immunity - is a state of resistance from infections.

Immune system - is a fluid complex network present in our body, comprising many independent cells, and provides defence mechanism or protect from millions of microbes or harmful compounds or antigens.

The immune system must be able to differentiate "self" from "*non self*". The human immune system consists of 3 lines defence.

The first line of defence provided by a set of mechanical, chemical and biological barriers. If the first line is breached / rupture / lysis, the second, third lines of barriers are activated, first is innate immune system, followed by acquired / adaptive immune system.



2. CELLS OF THE IMMUNE SYSTEM

The immune system depends upon the activities of three categories of white blood cells (WBCs) that are derived from bone marrow:

- ✓ ***Phagocytic cells:***
  Macrophages and dendritic cells are phagocytic cells that reside in the blood and tissues waiting to engulf foreign substances.
- ✓ ***T cells:***
  After leaving the bone marrow, some WBCs reach the thymus gland where they differentiate and become thymus-derived lymphocytes or T cells.
- ✓ ***B cells:***
  WBCs that do not reach the thymus gland become B lymphocytes or B cells

2.1. LYMPHATIC SYSTEM

Some of the WBCs migrate to guard peripheral tissues, some reside within the tissues, and others circulate in the blood stream and in a specialized system of vessels and nodules in the lymphatic system. The lymphatic system drains extracellular fluid and frees cells from tissues. The extracellular fluid and cells are transported through the body via the lymphatic vessels as lymph, and eventually emptied back into the blood system.

The lymphatic vessels closely parallel the body's veins and arteries. Lymph nodes are found throughout the lymphatic vessels and provide meeting areas for interaction between the immune system cells.

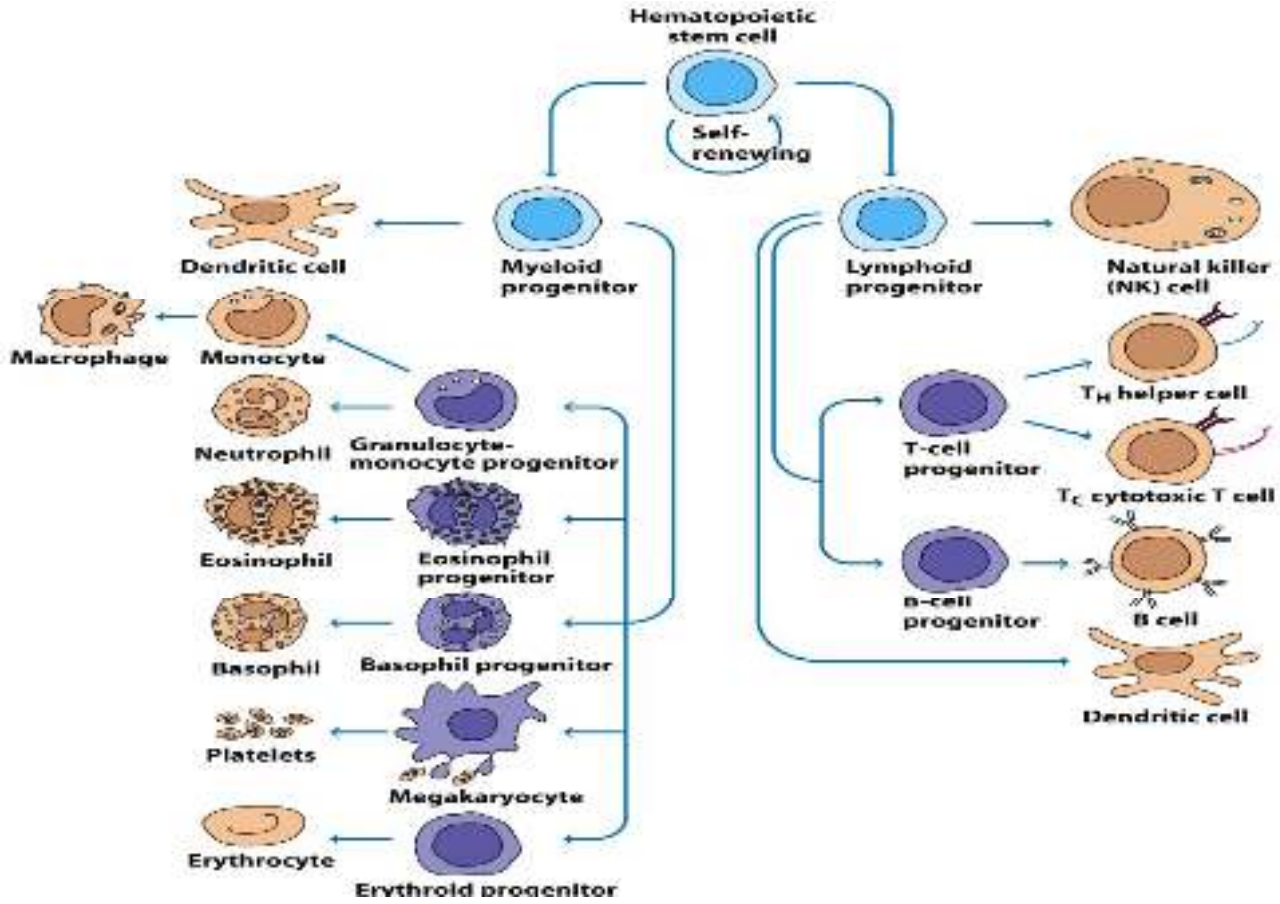
The lymphatic system contains the following:

Primary lymphoid organs:

- ❖ Bone marrow
- ❖ Thymus
- ❖ Lymphatic vessels

Secondary lymphoid organs

- ❖ Spleen
- ❖ Lymph node



The cells of immune system are:

1. Lymphocytes-

   a) T-lymphocytes
   b) B- lymphocytes
   c) NK cell

2. Phagocytic cells

   a) Monocytes
   b) Macrophages

3. Granulocytic cells

   a) Neutrophils
   b) Basophils
   c) Eosinophils

4. Dendritic cells

1. Lymphocytes:

- ✓ Lymphocytes are small, round cells found in peripheral blood, lymph, lymph nodes, lymphoid organs and in tissues.
- ✓ Lymphocytes represent 20-45% of total cells in peripheral blood and 99% of total cells in lymph and lymph node.
- ✓ According to side lymphocytes are divided into small (5-8μm), medium (8-12μm) and large (12-15μm).
- ✓ Depending on life span lymphocytes are classified into short lived (2 weeks) and long lived (3 years or more or even lifelong).

Broadly lymphocytes are divided into three sub-populations, on the basis of function and cell membrane components.

1. T-lymphocytes
2. B-lymphocytes
3. Natural killer cell

2. Phagocytic cells:

- ✓ Monocytes and macrophages are mononuclear phagocytic cells.
- ✓ Granulocyte-monocyte progenitor cell differentiates into promonocytes and neutrophil.
- ✓ Promonocytes leaves the bone marrow and enter into blood stream where they differentiate into mature monocytes.
- ✓ Monocytes circulate in blood for about 8 hours, during which they enlarge and then enter into tissues and differentiates into specific macrophages and dendritic cells.

1. Monocytes:

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

Immunological Functions of monocytes:

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

2. Macrophages:

- ✓ Monocyte migrates to tissue and differentiates into macrophages.
- ✓ Differentiation of monocytes into macrophages involves following changes:
- ✓ Cell enlarges 5-10 folds
- ✓ Intracellular granules increases in number and complexity
- ✓ Increase phagocytic ability
- ✓ Produces higher level of hydrolytic enzymes and cytokines
  - ▪ Macrophages serve different functions in different tissues.
  - ▪ Alveolar macrophages : in lungs
  - ▪ Histiocyte: connective tissue

- Kuffer cell: liver
- Messangial cell: kidney
- Microglial cell: brain
- Osteoclast: bone

Immunological functions of macrophages:

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

3. Granulocytic cells:

1. Neutrophil:

- ✓ Neutrophils are (11-14μm) in diameter with multilobed nucleus with granules in cytoplasm.
- ✓ It constitutes 50-70 % of total circulating WBC and remains for 7-8 hours in blood and then migrates to tissues
- ✓ Life span is 3-4 days.
- ✓ Also known as polymorphonuclear (PMN) leukocyte.
- ✓ Neutrophils are stained by both acidic and basic dye.

Immunological functions of Neutrophil:

- ✓ Phagocytic role in acute inflammatory response.
- ✓ It is the first immune cell to responds in inflammation.
- ✓ Basophils are non-phagocytic cell found in small number in blood and tissue
- ✓ Cytoplasm contains large number of prominent basophilic granules containing histamine, heparin, serotonin, and other hydrolytic enzymes
- ✓ Stained by basic dyes

Immunological functions:

- ✓ Provide anaphylactic and atopic allergic reaction

2. Eosinophils:

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

Immunological functions of Eosinophil:

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

3. Basophils:

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

Immunological functions:

- Provide anaphylactic and atopic allergic reaction

4. Dendritic cell:

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

Immunological functions:

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

3. TYPES OF IMMUNITY

Passive Immunity:

Antibodies from another person or animal that can be injected or transfused, Called passive because the individual did not create the antibodies, but instead received pre-formed antibodies.

Protection is effective, but duration is short lived and no memory is created.

Examples of passive immunity are maternal antibodies (trans-placental and breast milk) and injected antibodies (e.g., rabies, varicella, and tetanus immune globulins).

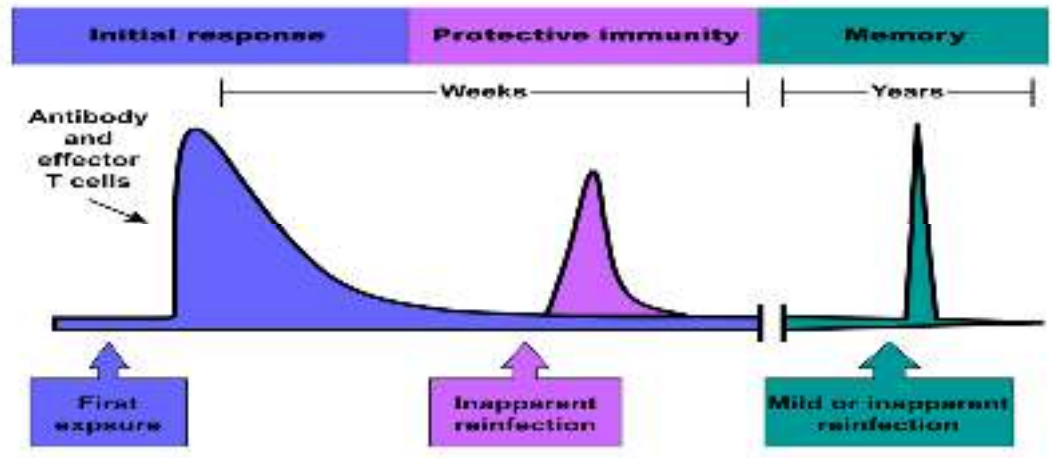
Active Immunity:

When the body is exposed to a foreign substance the cells of the immune system "actively" respond.

Active immunity is further divided into categories:

Innate Immunity - protective mechanisms we are born with

Aqcuired/Adaptive Immunity – cell mediated immunity (CMI) and humoral immunity (HI)



3.1. ACTIVE IMMUNITY

The body's immune response can be understood by following the course of an infection as described below:

- ✓ Most pathogens are kept outside of the body by protective mechanisms such as tears or skin that act as barriers.
- ✓ When there is an injury to tissue, bacteria or viruses can enter the tissue and cause infection. Innate cells (macrophages, dendritic cells) respond by recognizing viruses and bacteria as foreign and specialize in engulfing these invaders (phagocytosis). These innate cells and protective barriers are part of innate immunity because they "innately" respond to foreign substances.
- ✓ In addition, dendritic cells display the antigens on their cell surface and travel to the lymph nodes.
- ✓ In the lymph nodes the dendritic cells present the antigen to the T cells. The T cells then activate the B cells to make antibodies. The T cells and B cells are part of the adaptive immunity because they are "adapting" to the foreign substance and creating memory against future infections.

3.2. INNATE IMMUNITY – "FIRST" IMMUNE DEFENCE

Innate immunity consists of protective mechanisms we are born with, and are the first line of defence against anything recognized as non-self.

The produced immune response is not specific to the antigen and no memory of the antigen persists.
However, innate immunity is the crucial first step in most adaptive immune responses.

The following are the protective mechanisms of innate immunity (see Table):

- Physical and Chemical Mechanisms, Phagocytosis, Molecular Response, Inflammatory Response

Innate Immunity			
Physical and Chemical Mechanisms	**Phagocytosis**	**Molecular Response**	**Inflammatory Response**
Physical barriers: intact skin ✓ mucous ✓ membrane barrier (sneezing, coughing) ✓ cilia Chemical barriers: tears ✓ acid (pH) ✓ saliva ✓ bile	**Macrophages:** engulf and kill invading organisms **Dendritic cells:** ✓ engulf pathogen ✓ display antigen on cell surface ✓ travel to lymph node to present antigen to T cells ✓ Critical link between the innate and adaptive immune responses.	**Cytokines:** Cytokines are small proteins made by a cell that affect the behaviour of other cells. Examples: ✓ Cytokines cause vasodilation (heat and redness) ✓ Some types of interferon are antiviral cytokines which help healthy cells resist viral infection ✓ **Chemokines:** Chemokines are proteins secreted by macrophages that attract cells out of the blood stream and into the infected tissues.	The accumulation of fluid and cells at the site of infection causes the redness, swelling, heat, and pain known as inflammation. Inflammation is beneficial because it: recruits cells out of the blood stream, increases the flow of lymph to take away microbes and antigen bearing cells to the lymphoid tissue which will lead to adaptive immunity, and brings the T cells and B cells back to the site of infection.

		✓ **Complement:** The complement system is a group of approximately 20 proteins that coat bacterial surfaces and promote bacterial destruction by macrophages	

3.3. ACQUIRED / ADAPTIVE IMMUNITY - "SECOND" IMMUNE DEFENCE

Adaptive immunity is the second line of defence against anything recognized as non-self and it provides protection against re-exposure to the same pathogen.

Characteristics of adaptive immunity:

- ✓ Specificity: the immune response is specific to the antigen that produced it (E.g. antibody for measles antigen has no effect on rubella antigen)
- ✓ Tolerance: the immune response is able to differentiate between self and non self so that body tissues are not destroyed
- ✓ Memory: with subsequent exposure to an antigen there is a rapid and strong immune response. This is called an amnestic response.

Adaptive immunity is divided into two categories:

1. Cell mediated immunity
2. Humoral (antibody) immunity

3.3.1. CELL MEDIATED IMMUNITY

Cell mediated immunity describes any immune response where T cells have the main role.

B cells are not activated by most antigens without "help" from helper T cells. The activation of T cells is an essential first stage in virtually all adaptive immune responses. This is called the "T cell-dependent immune response".

T cells do not recognize microorganisms in the extracellular fluids. Instead, T cell receptors bind to fragments of antigens (epitopes) that are presented on the surface of antigen presenting cells (APC). There are three main types of APC:

- ❖ Macrophages
- ❖ Dendritic cells
- ❖ Naïve B cells

When T cells recognize an antigen presented by the APC, they can differentiate into several different types of T cells:

Cytotoxic T cells: Kill cells infected with intracellular pathogens such as viruses

Helper T cells:

- ✓ Activate antigen and stimulate B cells to differentiate and produce antibodies
- ✓ Activate macrophages to become more efficient at killing the pathogen
- ✓ Control intracellular bacterial infections (e.g. tuberculosis) that grow in intracellular membrane-bound vesicles of macrophages. The macrophages can't kill the bacteria but instead display the bacterial antigen on the surface so that it can be recognized by T cells

Regulatory T cells:

- ✓ Suppress lymphocytes and control the immune response

3.3.2. HUMORAL IMMUNITY

Humoral immunity is mediated by B cells. B cells react against foreign substances in the extracellular spaces of the body by producing and secreting antibodies (Abs). These Abs are present in the biological fluids of the body (the humours); hence the term humoral immunity.

Many microorganisms multiply in the extracellular spaces of the body, and most intracellular pathogens spread by moving from cell to cell through the extracellular fluids.

These extracellular spaces are protected by humoral immunity where antibodies either kill the extracellular organism and the intracellular organism as it is moving from cell to cell or bind the pathogen and present it to T cells.

B cells display immunoglobulin molecules (antibodies) on their surface membranes, which act as receptors for the antigens. B cell antibody receptors can either bind to helper T cells that have interacted with an APC or bind to extracellular microorganisms such as bacteria.

Once an antigen binds to an antibody with the best "fit", the B cell differentiates into plasma cells or B memory cells.

Plasma cells:

These cells operate as factories to manufacture the chosen antibody and then secrete those antibodies.

B memory cells:

These cells mediate immunological memory. They respond rapidly on reexposure to the antigen that originally induced them.

Differences between HI & CMI

Characteristics	Humoral Immunity	Cell Mediated Immunity
Definition	The aspect of immunity, mediated by macromolecules found in the extracellular body fluids is called humoral immunity.	The aspect of immunity that identifies and destroys infected cells is called cell mediated immunity.
Pathogens	The humoral immunity protects against extracellular pathogens.	The cell mediated immunity protects against intracellular pathogens.
Main cells	The main cells, involved in the humoral immunity are the B-cells. These cells are generated and mature in the bone marrow.	The main cells, involved in the cell mediated immunity are the T-cells. These cells are generated in the bone marrow and complete their development in the thymus.
Activation	The end result of the activation is the differentiation of plasma B-cells, secreting antibodies.	The end result of the activation is the secretion of cytokines.
Onset	Rapid	Delayed

ANTIBODIES

Classes of Antibodies – (D-GAME=Defensive GAME)

There are five classes of antibodies: IgM, IgG, IgA, IgD and IgE. Each class performs particular functions. The immune response to injected vaccines involves IgG and IgM. Antibodies as a class are known as immunoglobulins:

Immunoglobulin D (IgD)

- Constitutes only a very small fraction (0.2%) of immunoglobulin in the body
- Acts as an antigen receptor on the surfaces of B cells
- Unknown activity

Immunoglobulin G (IgG):

- The most abundant class of antibody, constituting approximately 80% of all antibodies in serum, produced slowly upon primary exposure to an antigen
- Produced rapidly during secondary or subsequent exposure, becoming the major antibody present
- The principal humoral component of immunological memory
- The only antibody that crosses the placenta.
- It helps protect the newborn from infection through passive immunity.

Immunoglobulin A (IgA):

- Represents approximately 10% to 20% of the immunoglobulins in serum
- Most abundant immunoglobulin in tissues
- Prevents or interferes with the attachment of viruses and bacteria to mucosa of respiratory and digestive systems
- Protects against enterotoxins released by certain bacteria; for example, forms an antibody-antigen complex with cholera toxin, preventing it from binding to specific receptors on the intestinal membrane
- Plays a role in eliminating food antigens from the circulatory system
- The main secretory immunoglobulin; found in exocrine secretions (e.g., breast• milk, saliva, tears, respiratory and digestive secretions, urine).

Immunoglobulin M (IgM):

- A valuable diagnostic marker for infectious disease because it is usually the first immunoglobulin made following Ag exposure and is relatively short-lived, effective in activating complement,
- Participates in the lysis (bursting apart) of cells,
- Generally remains in the blood; does not diffuse into the surrounding tissues due to its large size

Immunoglobulin E (IgE)

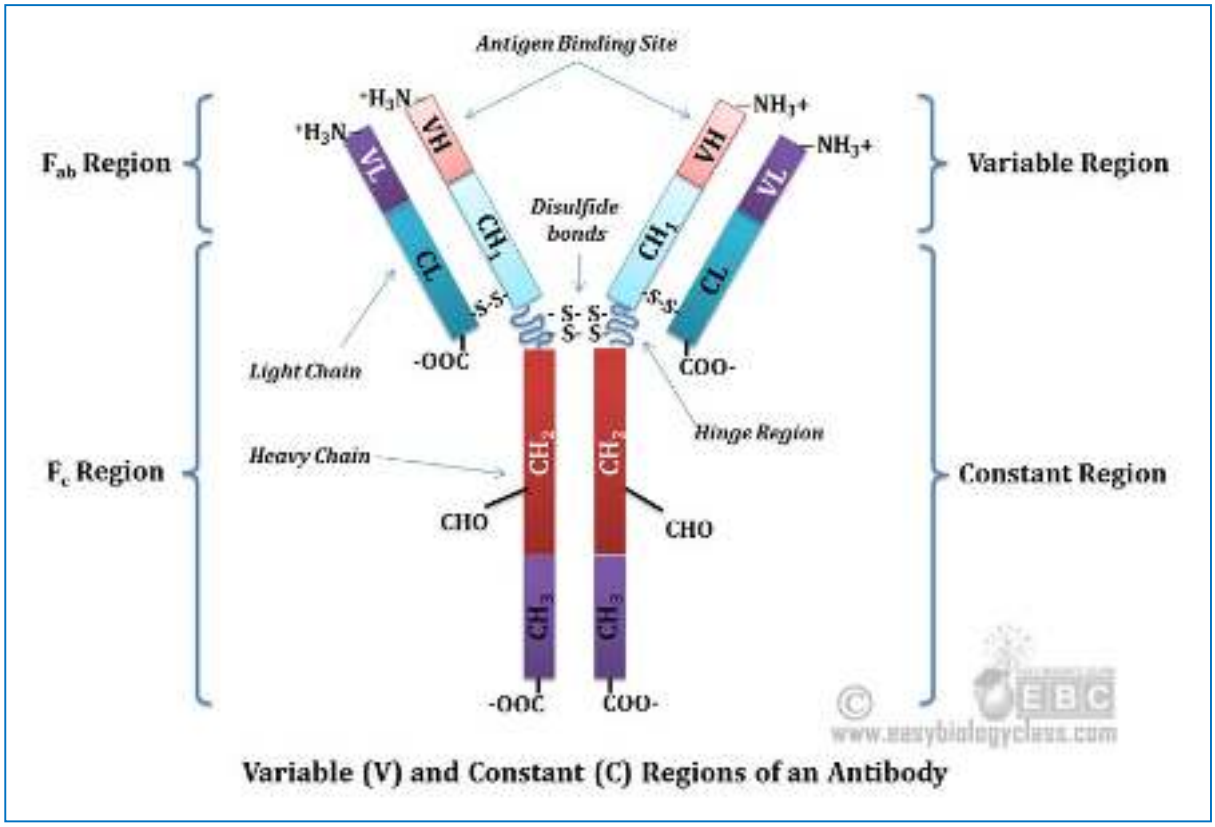
- Minute concentration in serum
- Involved in mediating allergic reactions
- Elevated in people with hypersensitivity to allergens, as well as those with eczema, asthma, or other respiratory problems, especially useful against parasitic infections (e.g., worms)

Properties of immunoglobulins

Property	***IgD***	***IgG***	***IgA***	***IgM***	***IgE***
Mol Wt	*180000*	*150000*	*160000*	*900000*	*190000*
Sedimentation coefficient	*7*	*7*	*7*	*19*	*8*
Heavy chain	*Delta*	*Gamma*	*Alpha*	*Mu*	*Epsilon*
Light chain	*K or L*	*K or L*	*K or L*	*K or L*	*K or L*
Serum con (mg/ml)	*0.03*	*12*	*2*	*1.2*	*0.00004*
Placental transport	-	+	-	-	-
Half life	*3 days*	*23 days*	*6-8days*	*5 days*	*2-3days*
Intravascular distribution (%)	*75*	*45*	*42*	*80*	*50*
Present in milk	-	+	+	-	-

Antibody function- Antibodies have three main functions:

1) *Neutralization:* Antibodies bind to pathogens (e.g., viruses, bacteria) and block their access to cells, then the antibody-antigen complex is engulfed by a macrophage.
2) *Opsonization:* Encapsulated bacteria (e.g., Hib, pnemococcal, and meningococcal) evade the innate immune system because they are not recognized by macrophages or dendritic cells. However, encapsulated bacteria can be recognized by antibodies. The antibody coats the bacteria to enable the ingestion by macrophages and dendritic cells through the process of opsonization.
3) *Complement Activation:* Antibodies bind to certain bacteria in the plasma. A region on the antibody is a receptor to complement proteins which will help lyse the bacteria or attract the macrophages to it.



Antibody – Structure

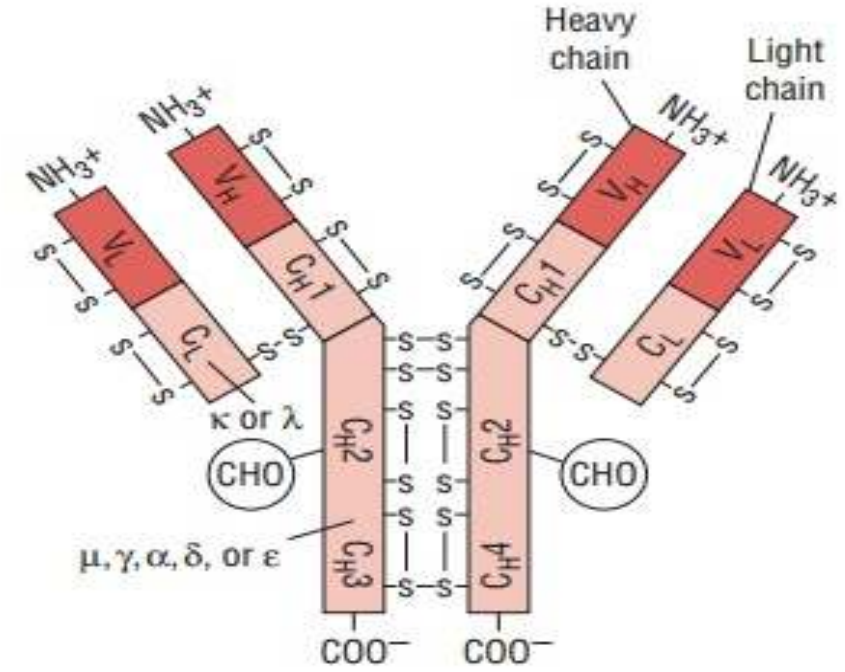


FIG. 13-1. Schematic diagram of monomer of the immunoglobulin.

					
	IgM	IgG	IgA	IgE	IgD
Heavy Chain	μ (mu)	γ (gamma)	α (alpha)	ε (epsilon)	δ (delta)
MW (Da)	900k	150k	385k	200k	180k
% of total antibody in serum	6%	80%	13%	0.002%	1%
Fixes complement	Yes	Yes	No	No	No
Function	Primary response, fixes complement. Monomer serves as B-cell receptor	Main blood antibody, neutralizes toxins, opsonization	Secreted into mucus, tears, saliva	Antibody of allergy and anti-parasitic activity	B cell Receptor

GLOSSARY –DEFINITIONS (FOR 2M)

Antigen

Antigens are the particles which are recognised by human body as foreign body / particle.
Antigens are stimulate the immune system.
Antigens are foreign / self particles

Antigens are:

Hetero antigen – are foreign particles, microbial agents (cell wall, flagella, toxins).
Non microbial agents – Pollengrains, allergens, animals, incompatible blood cells, transplanted organs & tissues.

Self antigen- cancer cells / tumor cells

Immunogenicity

The molecules / antigens can able to induce specific antibodies for immune response

Antigenecity

The molecule / antigens / particles cannot able to induce antibodies, they binds specifically high molecular weight proteins / antibodies for immune response

All immunogenic are antigens too…

All antigenic cannot be considered as immunogenic

Active immunity

When the body is exposed to a foreign substance the cells of the immune system "actively" respond

Adaptive immunity

The cells of the immune system "adapt" to the foreign substance and create memory against future infections.

Adjuvant

A substance added to a vaccine to enhance the immune response by intensity and/or duration.

Anamnestic response

A renewed rapid production of an antibody on the second (or subsequent) encounter with the same antigen

Antigen – micro, chemicals, allerges, food products & own cells

A foreign substance which, when introduced into the body is capable of inducing an immune response.

APC (antigen presenting cell)

Highly specialized cells that process antigens and display their peptide fragments on their surface

Cytotoxic T cells

Kill intracellular pathogens like viruses

Dendritic cells

Phagocytic cells found in most tissues. They act as antigen presenting cells (APC), travel to lymph nodes and present antigens to T cells

Epitope

The site on an antigen that is recognized by an antibody or an antigen receptor

Helper T cells

T cells that stimulate B cells to differentiate and produce antibodies Immunoglobulin A class of antibodies

Immunoprophylaxis

Disease prevention by immunologic means. Active immunoprophylaxis involves the administration of vaccines to stimulate the host's own immune system. Passive immunoprophylaxis involves the administration of immune globulins from an immune donor.

Innate immunity

Protective mechanisms we are born with e.g., cilia, skin, mucosal membranes Interferon Antiviral cytokines which help healthy cells resist viral infection

Lymph

Extracellular fluid that accumulates in tissues and is carried through the lymphatic system by the lymphatic vessels

Lysis

Process of disintegration or dissolution of cells

Macrophage

Large phagocytic cell; creates inflammation cytokines; acts as an antigen presenting cell (APC)

Memory cell

Lymphocytes that mediate immunological memory

Passive immunity

Antibodies from another person or animal are injected or transfused into an individual who receives "passive" immunity

Phagocytosis

The process by which a cell engulfs and digests solid substances

Plasma Cell

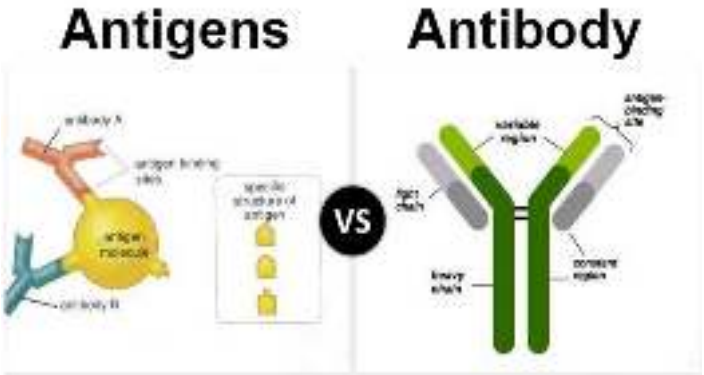
Specialized B cells that create antibodies

Regulatory T cells

Suppress lymphocytes and control the immune response

Differences between antigen and antibody

Antibodies are produced by the immune system in response to **antigens** (material perceived as foreign). The antibody response to a particular antigen is highly specific and often involves a physical association between the two molecules. This association is governed by biochemical and molecular forces. The reaction between antigens (Ags) and antibodies (Abs) involves complementary binding sites on the Ab and on the Ag molecules. Some of the differences between antigen and antibody are as follows:



S.No.	Characteristics	Antigen	Antibody
1	*Molecule Type*	Usually, proteins may also be polysaccharides, lipids or nucleic acids.	Proteins
2	*Definition*	These are substances that provoke an immune response.	These are Glycoproteins that are secreted by immune cells (plasma cells) in response to a foreign substance (antigen).
3	*Effect*	Cause disease or allergic reactions.	Protect the system by lysis of antigenic material.
4	*Origin*	Within the body or externally.	Within the body.
5	*Parts*	Highly variable with different structural conformations and is	Composed of three main parts: -Two light chains

		usually composed of different epitopes.	-Two heavy chains -Four polypeptides
6	*Prevalence*	Exists in all types of cells; mostly found in viruses, bacteria, and fungi.	Only present in some types of cells.
7	*Synonyms*	Immunogens	Immunoglobulins
8	*Specific binding site*	Epitope	Paratope
9	*Complexity*	Medium; exists due to random mutations in the cell's gene.	Very High; Complex chemical that bonds to a very specific Antigen.
10	*Source*	Usually from a foreign substance (viruses, and bacterial and fungal toxins).	Naturally produced by the body (B lymphocytes or B cells).
11	*Kinds*	There are three basic kinds of antigens (Exogenous, Endogenous, and Autoantigens)	There are five basic kinds of antibodies (IgG, IgM, IgA, IgE, and IgD).
12	*Examples*	**Exogenous antigens**: bacteria, viruses, fungi, etc. **Endogenous antigens:** Blood group antigens, HLA (Histocompatibility Leukocyte antigens), etc. **Autoantigens:** Nucleoproteins, Nucleic acids, etc.	Breast milk, tears, saliva, sweat, and mucus.

HYPERSENSITIVITY

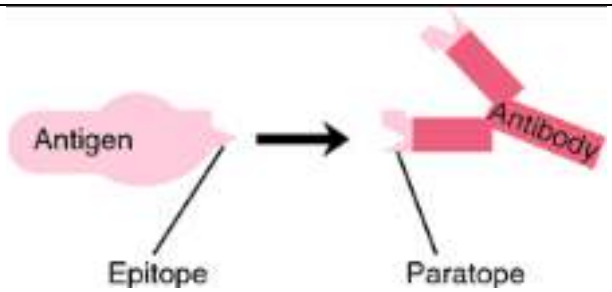
DEFINITIONs

Hypersensitivity is an immunological state in which the immune system **"over-reacts"** to foreign antigen such that the immune response itself is more harmful than the antigen

Hypersensitivity refers to excessive, undesirable (damaging, discomfort-producing and sometimes fatal) reactions produced by the normal immune system. Hypersensitivity reactions require a pre- sensitized (immune) state of the host. Immune response recruits and mobilizes a series of effectors molecules that induce a localized inflammatory response, which ultimately removes the antigen.

Hypersensitivity	Immune & inflammatory responses that are harmful to the host
Hypersensitivity reactions	Harmful antigen-specific immuneυ responses, occur when an individual who has been primed by an innocuous antigen subsequently encounters the same antigen, produce tissue injury and dysfunction.
Atopy	Genetic tendency to develop classic allergic diseases
Allergy	Damaging immune response by the body to a substance
Allergen	Substance causing allergic reaction

Immunogen	Substance that induces immune response.
Complete Antigen	Substance that induces immune response and reacts with products of immune response.
Incomplete Antigen	Substance that reacts with products of immune system but not induce an immune response
Hapten	Low molecular weight molecule that is recognized by preformed antibody but is not itself immunogenic unless conjugated to a carrier molecule that provides epitopes recognized by helper T cells.
Epitope	Antigenic structure recognized by antibody
Paratope	Paired heavy chain and light chain form antigen binding site



Hypersensitivity reactions: four types

Based on

- The mechanisms involved
- Time taken for the reaction

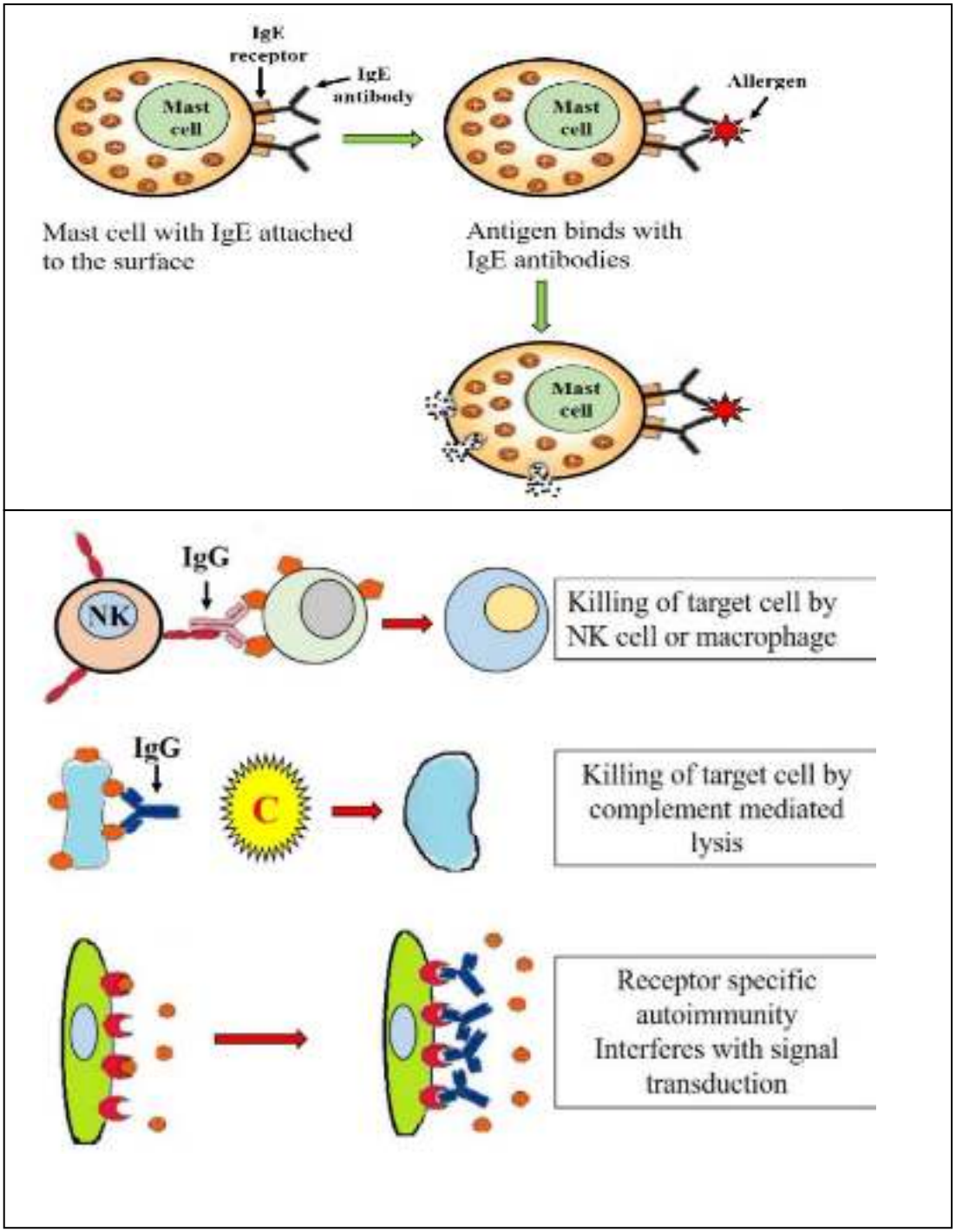
Classification of Hypersensitivity

1. TYPE I – Immediate, **A**topic, **A**naphylactic, **A**llergy (2-30 Minutes)
2. TYPE II – ANTI**B**ODY Dependant (5-8 hrs)
3. TYPE III – Immune **C**OMPLEX (2-8 hrs)
4. TYPE IV – CELL MEDIATED / **D**elayed Type of Hypersensitivity (24 hours to days)

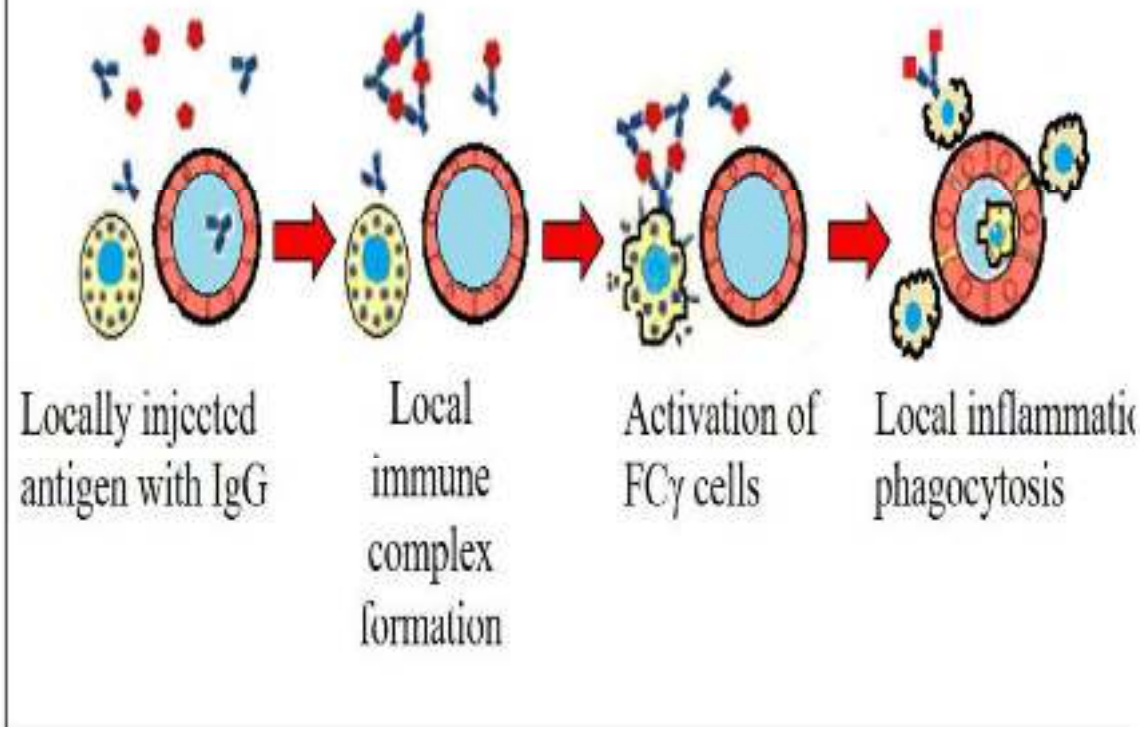
	Immune reactant	**Mechanism of activation**	**Ag form**	**Clinical syndrome**
TYPE I	IgE mediated HS	Ag induces cross linking of IgE bound to mast cells and basophils with release of vasoactive mediators	Soluble Ag	**A**topic, **A**naphylactic, **A**llergy, Hay fever, Asthma, Food allergies

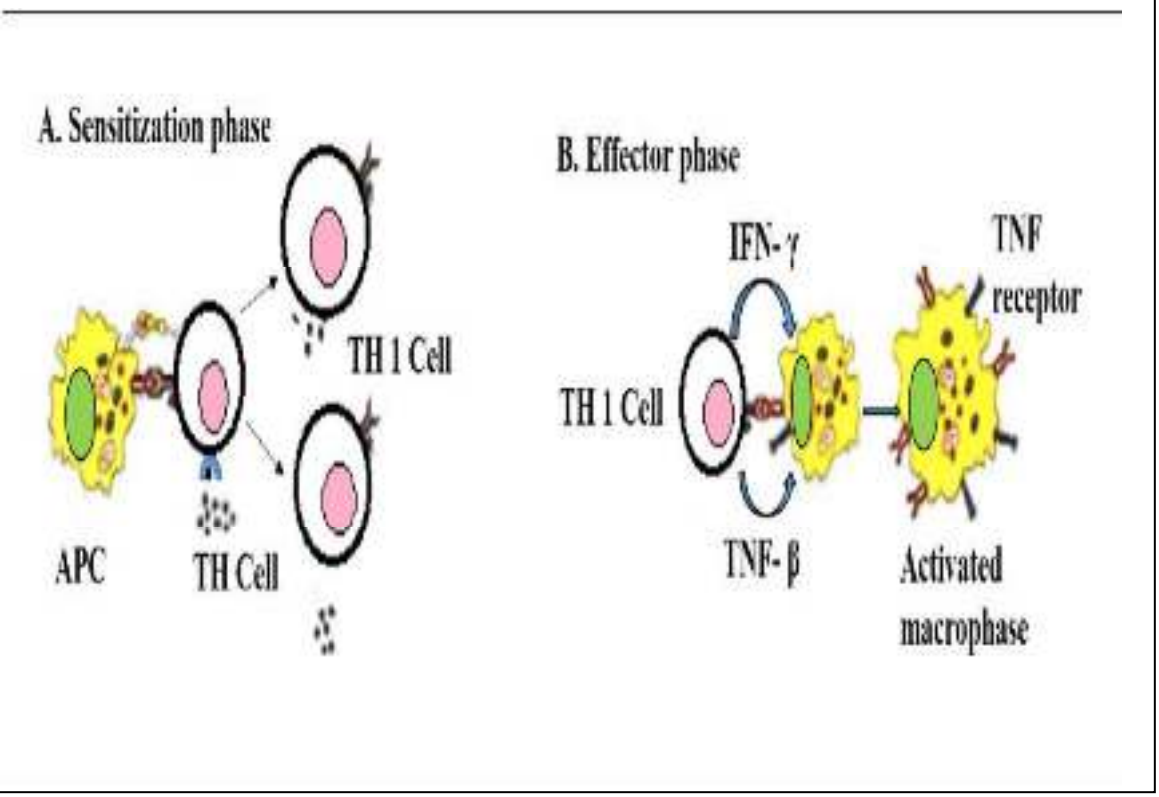
TYPE II	IgG cytotoxic HS IgG or IgM	Ab directed against cells surface antigens mediated cell destruction via complement activation	Cell bound Ag	Anti**b**ody mediated damage, Autoimmune haemolytic anemia, agranulocytosis, Erythroblastosis fetalis, Blood transfusion reactions
TYPE III	Immune **c**omplex mediated HS IgG or IgM	Ag-Ab complexes deposited in various tissues induce complement activation and an ensuring inflammatory response mediated by massive infiltration of neutrophils	Soluble Ag	Arthus reaction, Serum sickness, Glomerulonephritis, RA, systemic lupus
TYPE IV	Cell mediated HS T cells	Sensitized TH1 cells release cytokines that activate macrophages or TC cells which mediate direct cellular damage	Soluble Ag or Cell bound Ag	Tubercular lesions Contact dermatitis

Type I

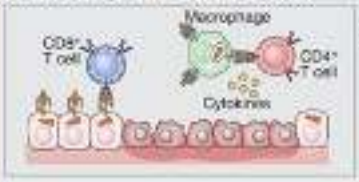

IgE receptor
IgE antibody
Mast cell
Allergen
Mast cell with IgE attached to the surface
Antigen binds with IgE antibodies
Mast cell
IgG
NK
Killing of target cell by NK cell or macrophage
IgG
C
Killing of target cell by complement mediated lysis
Receptor specific autoimmunity
Interferes with signal transduction

TYPE III


Locally injected antigen with IgG
Local immune complex formation
Activation of FCγ cells
Local inflammatic phagocytosis


A. Sensitization phase
TH 1 Cell
APC
TH Cell
B. Effector phase
IFN-γ
TNF receptor
TH 1 Cell
TNF-β
Activated macrophase

Types of Hypersensitivity Reactions

	Type of hypersensitivity	Pathologic immune mechanisms	Mechanisms of tissue injury and disease	
Type I	Immediate hypersensitivity (Type I)	T_H2 cells, IgE antibody, mast cells, eosinophils 	Mast cell-derived mediators (vasoactive amines, lipid mediators, cytokines) Cytokine-mediated inflammation (eosinophils, neutrophils)	Antibody mediated
Type II	Antibody-mediated diseases (Type II)	IgM, IgG antibodies against cell surface or extracellular matrix antigens 	Complement and Fc receptor-mediated recruitment and activation of leukocytes (neutrophils, macrophages) Opsonization and phagocytosis of cells Abnormalities in cellular function, e.g. hormone receptor signaling	
Type III	Immune complex-mediated diseases (Type III)	Immune complexes of circulating antigens and IgM or IgG antibodies deposited in vascular basement membrane 	Complement and Fc receptor-mediated recruitment and activation of leukocytes	
Type IV	T cell-mediated diseases (Type IV)	1. $CD4^+$ T cells (delayed type hypersensitivity) 2. $CD8^+$ CTLs (T cell-mediated cytolysis) 	1. Macrophage activation, cytokine-mediated inflammation 2. Direct target cell lysis, cytokine-mediated inflammation	T cell mediated

UNIT III - IMMUNOLOGY

- ✓ Principles of Immunity,
- ✓ Humoral immunity, cell mediated immunity,
- ✓ Antigen – antibody reactions,
- ✓ Hypersensitivity and its applications.
- ✓ Active & passive immunizations vaccine preparation, standardization & storage of BCG, cholera, smallpox, polio, typhus, tetanus toxoid, immuno serum & diagnostic agents.

1. INTRODUCTION

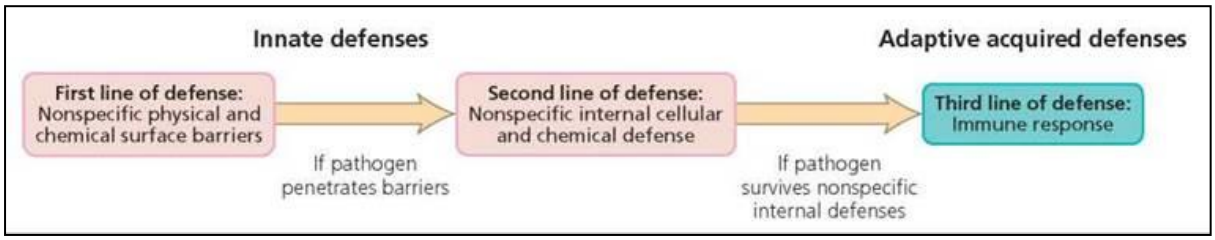
The body is protected from infectious agents and other harmful substances by a variety of cells and molecules that make up the immune system. Immunity is the ability of the human body to tolerate the presence of material indigenous to the body (self), and to eliminate foreign (non-self) material. Foreign substances such as viruses, bacteria, toxins, and parasites are surrounded by antigens that, when introduced into the body, are capable of inducing a response by the immune system. This discriminatory ability provides protection from infectious disease, since most agents or associated toxins are identified as foreign by the immune system.

Immunity - is a state of resistance from infections.

Immune system - is a fluid complex network present in our body, comprising many independent cells, and provides defence mechanism or protect from millions of microbes or harmful compounds or antigens.

The immune system must be able to differentiate "self" from "*non self*". The human immune system consists of 3 lines defence.

The first line of defence provided by a set of mechanical, chemical and biological barriers. If the first line is breached / rupture / lysis, the second, third lines of barriers are activated, first is innate immune system, followed by acquired / adaptive immune system.



2. CELLS OF THE IMMUNE SYSTEM

The immune system depends upon the activities of three categories of white blood cells (WBCs) that are derived from bone marrow:

- ✓ ***Phagocytic cells:***
  Macrophages and dendritic cells are phagocytic cells that reside in the blood and tissues waiting to engulf foreign substances.
- ✓ ***T cells:***
  After leaving the bone marrow, some WBCs reach the thymus gland where they differentiate and become thymus-derived lymphocytes or T cells.
- ✓ ***B cells:***
  WBCs that do not reach the thymus gland become B lymphocytes or B cells

2.1. LYMPHATIC SYSTEM

Some of the WBCs migrate to guard peripheral tissues, some reside within the tissues, and others circulate in the blood stream and in a specialized system of vessels and nodules in the lymphatic system. The lymphatic system drains extracellular fluid and frees cells from tissues. The extracellular fluid and cells are transported through the body via the lymphatic vessels as lymph, and eventually emptied back into the blood system.

The lymphatic vessels closely parallel the body's veins and arteries. Lymph nodes are found throughout the lymphatic vessels and provide meeting areas for interaction between the immune system cells.

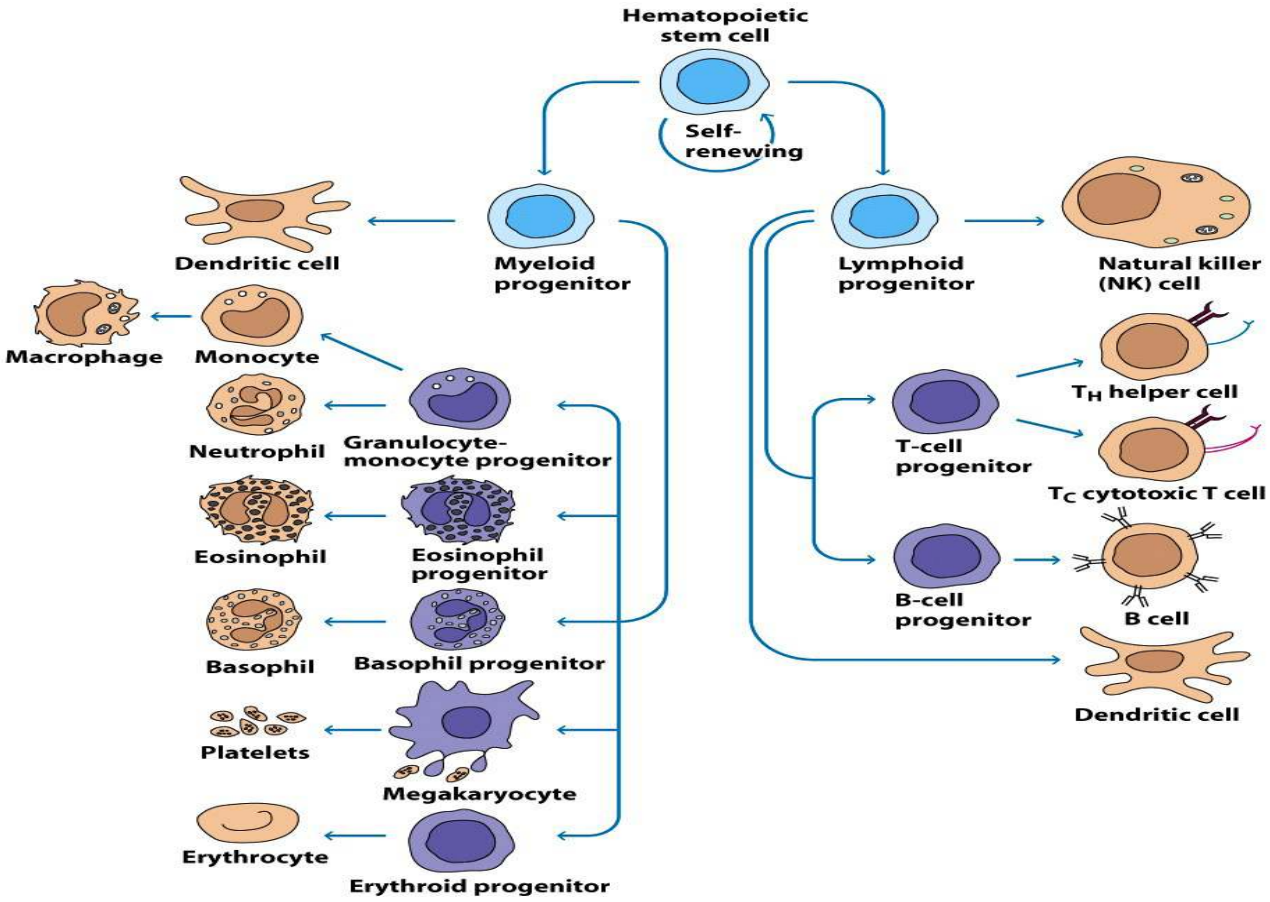
The lymphatic system contains the following:

Primary lymphoid organs:

- ❖ Bone marrow
- ❖ Thymus
- ❖ Lymphatic vessels

Secondary lymphoid organs

- ❖ Spleen
- ❖ Lymph node



The cells of immune system are:

1. Lymphocytes-

   a) T-lymphocytes
   b) B- lymphocytes
   c) NK cell

2. Phagocytic cells

   a) Monocytes
   b) Macrophages

3. Granulocytic cells

   a) Neutrophils
   b) Basophils
   c) Eosinophils

4. Dendritic cells

1. Lymphocytes:

- ✓ Lymphocytes are small, round cells found in peripheral blood, lymph, lymph nodes, lymphoid organs and in tissues.
- ✓ Lymphocytes represent 20-45% of total cells in peripheral blood and 99% of total cells in lymph and lymph node.
- ✓ According to side lymphocytes are divided into small (5-8μm), medium (8-12μm) and large (12-15μm).
- ✓ Depending on life span lymphocytes are classified into short lived (2 weeks) and long lived (3 years or more or even lifelong).

Broadly lymphocytes are divided into three sub-populations, on the basis of function and cell membrane components.

1. T-lymphocytes
2. B-lymphocytes
3. Natural killer cell

2. Phagocytic cells:

- ✓ Monocytes and macrophages are mononuclear phagocytic cells.
- ✓ Granulocyte-monocyte progenitor cell differentiates into promonocytes and neutrophil.
- ✓ Promonocytes leaves the bone marrow and enter into blood stream where they differentiate into mature monocytes.
- ✓ Monocytes circulate in blood for about 8 hours, during which they enlarge and then enter into tissues and differentiates into specific macrophages and dendritic cells.

1. Monocytes:

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

Immunological Functions of monocytes:

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

2. Macrophages:

- ✓ Monocyte migrates to tissue and differentiates into macrophages.
- ✓ Differentiation of monocytes into macrophages involves following changes:
- ✓ Cell enlarges 5-10 folds
- ✓ Intracellular granules increases in number and complexity
- ✓ Increase phagocytic ability
- ✓ Produces higher level of hydrolytic enzymes and cytokines
  - ▪ Macrophages serve different functions in different tissues.
  - ▪ Alveolar macrophages : in lungs
  - ▪ Histiocyte: connective tissue

- Kuffer cell: liver
- Messangial cell: kidney
- Microglial cell: brain
- Osteoclast: bone

Immunological functions of macrophages:

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

3. Granulocytic cells:

1. Neutrophil:

- ✓ Neutrophils are (11-14μm) in diameter with multilobed nucleus with granules in cytoplasm.
- ✓ It constitutes 50-70 % of total circulating WBC and remains for 7-8 hours in blood and then migrates to tissues
- ✓ Life span is 3-4 days.
- ✓ Also known as polymorphonuclear (PMN) leukocyte.
- ✓ Neutrophils are stained by both acidic and basic dye.

Immunological functions of Neutrophil:

- ✓ Phagocytic role in acute inflammatory response.
- ✓ It is the first immune cell to responds in inflammation.
- ✓ Basophils are non-phagocytic cell found in small number in blood and tissue
- ✓ Cytoplasm contains large number of prominent basophilic granules containing histamine, heparin, serotonin, and other hydrolytic enzymes
- ✓ Stained by basic dyes

Immunological functions:

- ✓ Provide anaphylactic and atopic allergic reaction

2. Eosinophils:

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

Immunological functions of Eosinophil:

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

3. Basophils:

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

Immunological functions:

- Provide anaphylactic and atopic allergic reaction

4. Dendritic cell:

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

Immunological functions:

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

3. TYPES OF IMMUNITY

Passive Immunity:

Antibodies from another person or animal that can be injected or transfused, Called passive because the individual did not create the antibodies, but instead received pre-formed antibodies.

Protection is effective, but duration is short lived and no memory is created.

Examples of passive immunity are maternal antibodies (trans-placental and breast milk) and injected antibodies (e.g., rabies, varicella, and tetanus immune globulins).

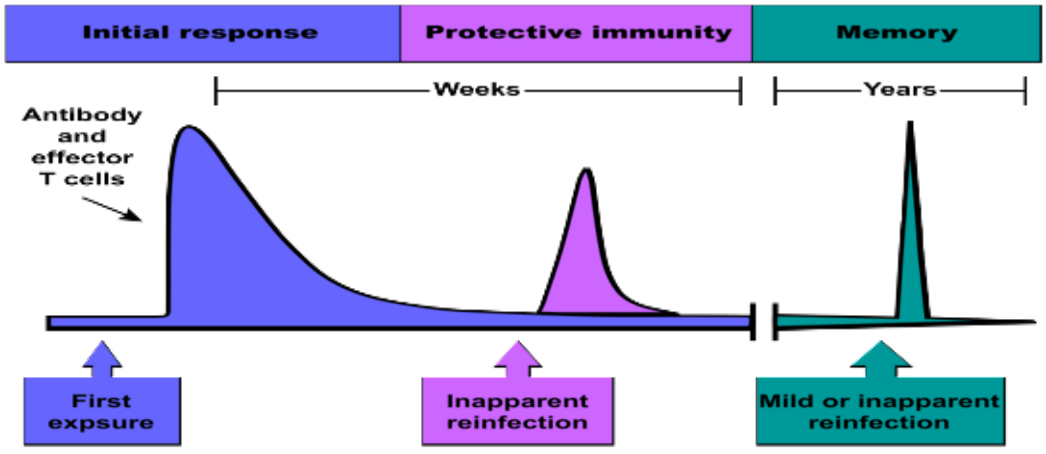
Active Immunity:

When the body is exposed to a foreign substance the cells of the immune system "actively" respond.

Active immunity is further divided into categories:

Innate Immunity - protective mechanisms we are born with

Aqcuired/Adaptive Immunity – cell mediated immunity (CMI) and humoral immunity (HI)



3.1. ACTIVE IMMUNITY

The body's immune response can be understood by following the course of an infection as described below:

- ✓ Most pathogens are kept outside of the body by protective mechanisms such as tears or skin that act as barriers.
- ✓ When there is an injury to tissue, bacteria or viruses can enter the tissue and cause infection. Innate cells (macrophages, dendritic cells) respond by recognizing viruses and bacteria as foreign and specialize in engulfing these invaders (phagocytosis). These innate cells and protective barriers are part of innate immunity because they "innately" respond to foreign substances.
- ✓ In addition, dendritic cells display the antigens on their cell surface and travel to the lymph nodes.
- ✓ In the lymph nodes the dendritic cells present the antigen to the T cells. The T cells then activate the B cells to make antibodies. The T cells and B cells are part of the adaptive immunity because they are "adapting" to the foreign substance and creating memory against future infections.

3.2. INNATE IMMUNITY – "FIRST" IMMUNE DEFENCE

Innate immunity consists of protective mechanisms we are born with, and are the first line of defence against anything recognized as non-self.

The produced immune response is not specific to the antigen and no memory of the antigen persists.
However, innate immunity is the crucial first step in most adaptive immune responses.

The following are the protective mechanisms of innate immunity (see Table):

- Physical and Chemical Mechanisms, Phagocytosis, Molecular Response, Inflammatory Response

Innate Immunity			
Physical and Chemical Mechanisms	**Phagocytosis**	**Molecular Response**	**Inflammatory Response**
Physical barriers: intact skin ✓ mucous ✓ membrane barrier (sneezing, coughing) ✓ cilia Chemical barriers: tears ✓ acid (pH) ✓ saliva ✓ bile	**Macrophages:** engulf and kill invading organisms **Dendritic cells:** ✓ engulf pathogen ✓ display antigen on cell surface ✓ travel to lymph node to present antigen to T cells ✓ Critical link between the innate and adaptive immune responses.	**Cytokines:** Cytokines are small proteins made by a cell that affect the behaviour of other cells. Examples: ✓ Cytokines cause vasodilation (heat and redness) ✓ Some types of interferon are antiviral cytokines which help healthy cells resist viral infection ✓ **Chemokines:** Chemokines are proteins secreted by macrophages that attract cells out of the blood stream and into the infected tissues.	The accumulation of fluid and cells at the site of infection causes the redness, swelling, heat, and pain known as inflammation. Inflammation is beneficial because it: recruits cells out of the blood stream, increases the flow of lymph to take away microbes and antigen bearing cells to the lymphoid tissue which will lead to adaptive immunity, and brings the T cells and B cells back to the site of infection.

		✓ **Complement:** The complement system is a group of approximately 20 proteins that coat bacterial surfaces and promote bacterial destruction by macrophages	

3.3. ACQUIRED / ADAPTIVE IMMUNITY - "SECOND" IMMUNE DEFENCE

Adaptive immunity is the second line of defence against anything recognized as non-self and it provides protection against re-exposure to the same pathogen.

Characteristics of adaptive immunity:

- ✓ Specificity: the immune response is specific to the antigen that produced it (E.g. antibody for measles antigen has no effect on rubella antigen)
- ✓ Tolerance: the immune response is able to differentiate between self and non self so that body tissues are not destroyed
- ✓ Memory: with subsequent exposure to an antigen there is a rapid and strong immune response. This is called an amnestic response.

Adaptive immunity is divided into two categories:

1. Cell mediated immunity
2. Humoral (antibody) immunity

3.3.1. CELL MEDIATED IMMUNITY

Cell mediated immunity describes any immune response where T cells have the main role.

B cells are not activated by most antigens without "help" from helper T cells. The activation of T cells is an essential first stage in virtually all adaptive immune responses. This is called the "T cell-dependent immune response".

T cells do not recognize microorganisms in the extracellular fluids. Instead, T cell receptors bind to fragments of antigens (epitopes) that are presented on the surface of antigen presenting cells (APC). There are three main types of APC:

- ❖ Macrophages
- ❖ Dendritic cells
- ❖ Naïve B cells

When T cells recognize an antigen presented by the APC, they can differentiate into several different types of T cells:

Cytotoxic T cells: Kill cells infected with intracellular pathogens such as viruses

Helper T cells:

- ✓ Activate antigen and stimulate B cells to differentiate and produce antibodies
- ✓ Activate macrophages to become more efficient at killing the pathogen
- ✓ Control intracellular bacterial infections (e.g. tuberculosis) that grow in intracellular membrane-bound vesicles of macrophages. The macrophages can't kill the bacteria but instead display the bacterial antigen on the surface so that it can be recognized by T cells

Regulatory T cells:

- ✓ Suppress lymphocytes and control the immune response

3.3.2. HUMORAL IMMUNITY

Humoral immunity is mediated by B cells. B cells react against foreign substances in the extracellular spaces of the body by producing and secreting antibodies (Abs). These Abs are present in the biological fluids of the body (the humours); hence the term humoral immunity.

Many microorganisms multiply in the extracellular spaces of the body, and most intracellular pathogens spread by moving from cell to cell through the extracellular fluids.

These extracellular spaces are protected by humoral immunity where antibodies either kill the extracellular organism and the intracellular organism as it is moving from cell to cell or bind the pathogen and present it to T cells.

B cells display immunoglobulin molecules (antibodies) on their surface membranes, which act as receptors for the antigens. B cell antibody receptors can either bind to helper T cells that have interacted with an APC or bind to extracellular microorganisms such as bacteria.

Once an antigen binds to an antibody with the best "fit", the B cell differentiates into plasma cells or B memory cells.

Plasma cells:

These cells operate as factories to manufacture the chosen antibody and then secrete those antibodies.

B memory cells:

These cells mediate immunological memory. They respond rapidly on reexposure to the antigen that originally induced them.

Differences between HI & CMI

Characteristics	Humoral Immunity	Cell Mediated Immunity
Definition	The aspect of immunity, mediated by macromolecules found in the extracellular body fluids is called humoral immunity.	The aspect of immunity that identifies and destroys infected cells is called cell mediated immunity.
Pathogens	The humoral immunity protects against extracellular pathogens.	The cell mediated immunity protects against intracellular pathogens.
Main cells	The main cells, involved in the humoral immunity are the B-cells. These cells are generated and mature in the bone marrow.	The main cells, involved in the cell mediated immunity are the T-cells. These cells are generated in the bone marrow and complete their development in the thymus.
Activation	The end result of the activation is the differentiation of plasma B-cells, secreting antibodies.	The end result of the activation is the secretion of cytokines.
Onset	Rapid	Delayed

ANTIBODIES

Classes of Antibodies – (D-GAME=Defensive GAME)

There are five classes of antibodies: IgM, IgG, IgA, IgD and IgE. Each class performs particular functions. The immune response to injected vaccines involves IgG and IgM. Antibodies as a class are known as immunoglobulins:

Immunoglobulin D (IgD)

- Constitutes only a very small fraction (0.2%) of immunoglobulin in the body
- Acts as an antigen receptor on the surfaces of B cells
- Unknown activity

Immunoglobulin G (IgG):

- The most abundant class of antibody, constituting approximately 80% of all antibodies in serum, produced slowly upon primary exposure to an antigen
- Produced rapidly during secondary or subsequent exposure, becoming the major antibody present
- The principal humoral component of immunological memory
- The only antibody that crosses the placenta.
- It helps protect the newborn from infection through passive immunity.

Immunoglobulin A (IgA):

- Represents approximately 10% to 20% of the immunoglobulins in serum
- Most abundant immunoglobulin in tissues
- Prevents or interferes with the attachment of viruses and bacteria to mucosa of respiratory and digestive systems
- Protects against enterotoxins released by certain bacteria; for example, forms an antibody-antigen complex with cholera toxin, preventing it from binding to specific receptors on the intestinal membrane
- Plays a role in eliminating food antigens from the circulatory system
- The main secretory immunoglobulin; found in exocrine secretions (e.g., breast• milk, saliva, tears, respiratory and digestive secretions, urine).

Immunoglobulin M (IgM):

- A valuable diagnostic marker for infectious disease because it is usually the first immunoglobulin made following Ag exposure and is relatively short-lived, effective in activating complement,
- Participates in the lysis (bursting apart) of cells,
- Generally remains in the blood; does not diffuse into the surrounding tissues due to its large size

Immunoglobulin E (IgE)

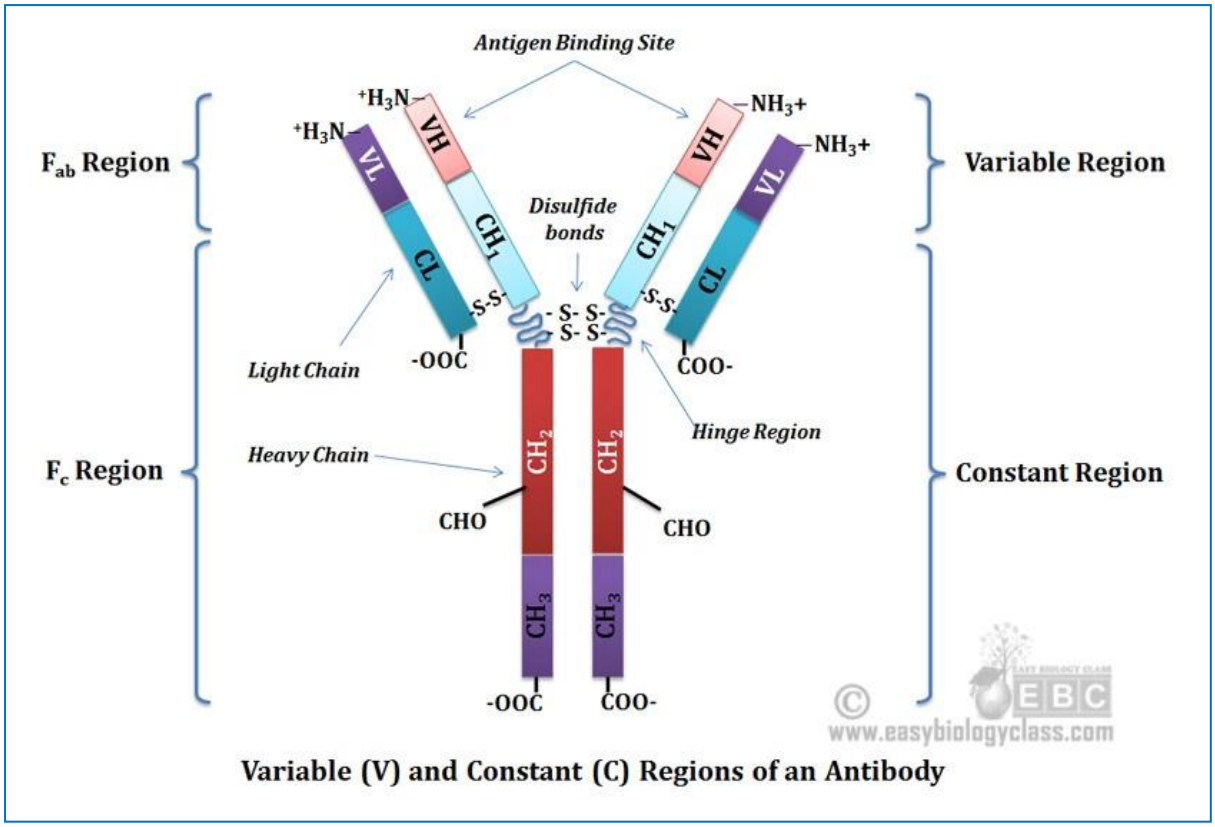
- Minute concentration in serum
- Involved in mediating allergic reactions
- Elevated in people with hypersensitivity to allergens, as well as those with eczema, asthma, or other respiratory problems, especially useful against parasitic infections (e.g., worms)

Properties of immunoglobulins

Property	***IgD***	***IgG***	***IgA***	***IgM***	***IgE***
Mol Wt	*180000*	*150000*	*160000*	*900000*	*190000*
Sedimentation coefficient	*7*	*7*	*7*	*19*	*8*
Heavy chain	*Delta*	*Gamma*	*Alpha*	*Mu*	*Epsilon*
Light chain	*K or L*	*K or L*	*K or L*	*K or L*	*K or L*
Serum con (mg/ml)	*0.03*	*12*	*2*	*1.2*	*0.00004*
Placental transport	-	+	-	-	-
Half life	*3 days*	*23 days*	*6-8days*	*5 days*	*2-3days*
Intravascular distribution (%)	*75*	*45*	*42*	*80*	*50*
Present in milk	-	+	+	-	-

Antibody function- Antibodies have three main functions:

1) *Neutralization:* Antibodies bind to pathogens (e.g., viruses, bacteria) and block their access to cells, then the antibody-antigen complex is engulfed by a macrophage.
2) *Opsonization:* Encapsulated bacteria (e.g., Hib, pnemococcal, and meningococcal) evade the innate immune system because they are not recognized by macrophages or dendritic cells. However, encapsulated bacteria can be recognized by antibodies. The antibody coats the bacteria to enable the ingestion by macrophages and dendritic cells through the process of opsonization.
3) *Complement Activation:* Antibodies bind to certain bacteria in the plasma. A region on the antibody is a receptor to complement proteins which will help lyse the bacteria or attract the macrophages to it.



Variable (V) and Constant (C) Regions of an Antibody

Antibody – Structure

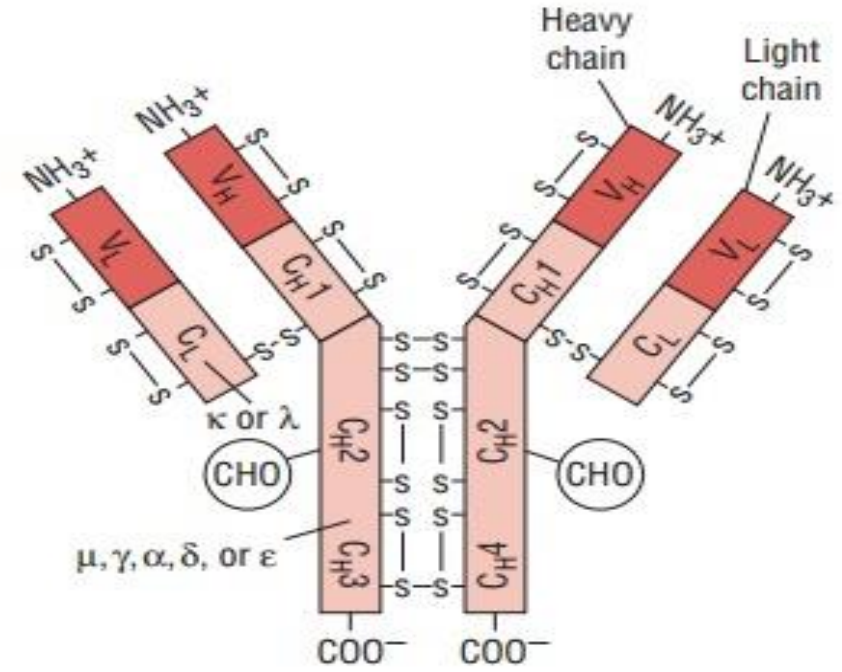


FIG. 13-1. Schematic diagram of monomer of the immunoglobulin.

					
	IgM	IgG	IgA	IgE	IgD
Heavy Chain	μ (mu)	γ (gamma)	α (alpha)	ε (epsilon)	δ (delta)
MW (Da)	900k	150k	385k	200k	180k
% of total antibody in serum	6%	80%	13%	0.002%	1%
Fixes complement	Yes	Yes	No	No	No
Function	Primary response, fixes complement. Monomer serves as B-cell receptor	Main blood antibody, neutralizes toxins, opsonization	Secreted into mucus, tears, saliva	Antibody of allergy and anti-parasitic activity	B cell Receptor

GLOSSARY –DEFINITIONS (FOR 2M)

Antigen

Antigens are the particles which are recognised by human body as foreign body / particle.
Antigens are stimulate the immune system.
Antigens are foreign / self particles

Antigens are:

Hetero antigen – are foreign particles, microbial agents (cell wall, flagella, toxins).
Non microbial agents – Pollengrains, allergens, animals, incompatible blood cells, transplanted organs & tissues.

Self antigen- cancer cells / tumor cells

Immunogenicity

The molecules / antigens can able to induce specific antibodies for immune response

Antigenecity

The molecule / antigens / particles cannot able to induce antibodies, they binds specifically high molecular weight proteins / antibodies for immune response

All immunogenic are antigens too…

All antigenic cannot be considered as immunogenic

Active immunity

When the body is exposed to a foreign substance the cells of the immune system "actively" respond

Adaptive immunity

The cells of the immune system "adapt" to the foreign substance and create memory against future infections.

Adjuvant

A substance added to a vaccine to enhance the immune response by intensity and/or duration.

Anamnestic response

A renewed rapid production of an antibody on the second (or subsequent) encounter with the same antigen

Antigen – micro, chemicals, allerges, food products & own cells

A foreign substance which, when introduced into the body is capable of inducing an immune response.

APC (antigen presenting cell)

Highly specialized cells that process antigens and display their peptide fragments on their surface

Cytotoxic T cells

Kill intracellular pathogens like viruses

Dendritic cells

Phagocytic cells found in most tissues. They act as antigen presenting cells (APC), travel to lymph nodes and present antigens to T cells

Epitope

The site on an antigen that is recognized by an antibody or an antigen receptor

Helper T cells

T cells that stimulate B cells to differentiate and produce antibodies Immunoglobulin A class of antibodies

Immunoprophylaxis

Disease prevention by immunologic means. Active immunoprophylaxis involves the administration of vaccines to stimulate the host's own immune system. Passive immunoprophylaxis involves the administration of immune globulins from an immune donor.

Innate immunity

Protective mechanisms we are born with e.g., cilia, skin, mucosal membranes Interferon Antiviral cytokines which help healthy cells resist viral infection

Lymph

Extracellular fluid that accumulates in tissues and is carried through the lymphatic system by the lymphatic vessels

Lysis

Process of disintegration or dissolution of cells

Macrophage

Large phagocytic cell; creates inflammation cytokines; acts as an antigen presenting cell (APC)

Memory cell

Lymphocytes that mediate immunological memory

Passive immunity

Antibodies from another person or animal are injected or transfused into an individual who receives "passive" immunity

Phagocytosis

The process by which a cell engulfs and digests solid substances

Plasma Cell

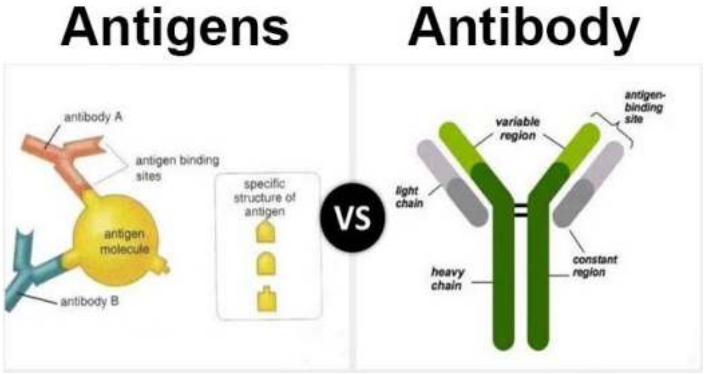
Specialized B cells that create antibodies

Regulatory T cells

Suppress lymphocytes and control the immune response

Differences between antigen and antibody

Antibodies are produced by the immune system in response to **antigens** (material perceived as foreign). The antibody response to a particular antigen is highly specific and often involves a physical association between the two molecules. This association is governed by biochemical and molecular forces. The reaction between antigens (Ags) and antibodies (Abs) involves complementary binding sites on the Ab and on the Ag molecules. Some of the differences between antigen and antibody are as follows:



S.No.	Characteristics	Antigen	Antibody
1	*Molecule Type*	Usually, proteins may also be polysaccharides, lipids or nucleic acids.	Proteins
2	*Definition*	These are substances that provoke an immune response.	These are Glycoproteins that are secreted by immune cells (plasma cells) in response to a foreign substance (antigen).
3	*Effect*	Cause disease or allergic reactions.	Protect the system by lysis of antigenic material.
4	*Origin*	Within the body or externally.	Within the body.
5	*Parts*	Highly variable with different structural conformations and is	Composed of three main parts: -Two light chains

		usually composed of different epitopes.	-Two heavy chains -Four polypeptides
6	*Prevalence*	Exists in all types of cells; mostly found in viruses, bacteria, and fungi.	Only present in some types of cells.
7	*Synonyms*	Immunogens	Immunoglobulins
8	*Specific binding site*	Epitope	Paratope
9	*Complexity*	Medium; exists due to random mutations in the cell's gene.	Very High; Complex chemical that bonds to a very specific Antigen.
10	*Source*	Usually from a foreign substance (viruses, and bacterial and fungal toxins).	Naturally produced by the body (B lymphocytes or B cells).
11	*Kinds*	There are three basic kinds of antigens (Exogenous, Endogenous, and Autoantigens)	There are five basic kinds of antibodies (IgG, IgM, IgA, IgE, and IgD).
12	*Examples*	**Exogenous antigens**: bacteria, viruses, fungi, etc. **Endogenous antigens:** Blood group antigens, HLA (Histocompatibility Leukocyte antigens), etc. **Autoantigens:** Nucleoproteins, Nucleic acids, etc.	Breast milk, tears, saliva, sweat, and mucus.

HYPERSENSITIVITY

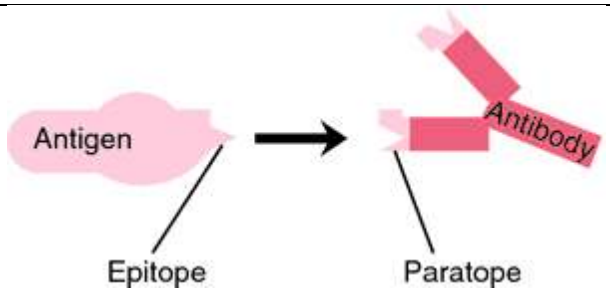
DEFINITIONs

Hypersensitivity is an immunological state in which the immune system **"over-reacts"** to foreign antigen such that the immune response itself is more harmful than the antigen

Hypersensitivity refers to excessive, undesirable (damaging, discomfort-producing and sometimes fatal) reactions produced by the normal immune system. Hypersensitivity reactions require a pre- sensitized (immune) state of the host. Immune response recruits and mobilizes a series of effectors molecules that induce a localized inflammatory response, which ultimately removes the antigen.

Hypersensitivity	Immune & inflammatory responses that are harmful to the host
Hypersensitivity reactions	Harmful antigen-specific immuneυ responses, occur when an individual who has been primed by an innocuous antigen subsequently encounters the same antigen, produce tissue injury and dysfunction.
Atopy	Genetic tendency to develop classic allergic diseases
Allergy	Damaging immune response by the body to a substance
Allergen	Substance causing allergic reaction

Immunogen	Substance that induces immune response.
Complete Antigen	Substance that induces immune response and reacts with products of immune response.
Incomplete Antigen	Substance that reacts with products of immune system but not induce an immune response
Hapten	Low molecular weight molecule that is recognized by preformed antibody but is not itself immunogenic unless conjugated to a carrier molecule that provides epitopes recognized by helper T cells.
Epitope	Antigenic structure recognized by antibody
Paratope	Paired heavy chain and light chain form antigen binding site



Hypersensitivity reactions: four types

Based on

- The mechanisms involved
- Time taken for the reaction

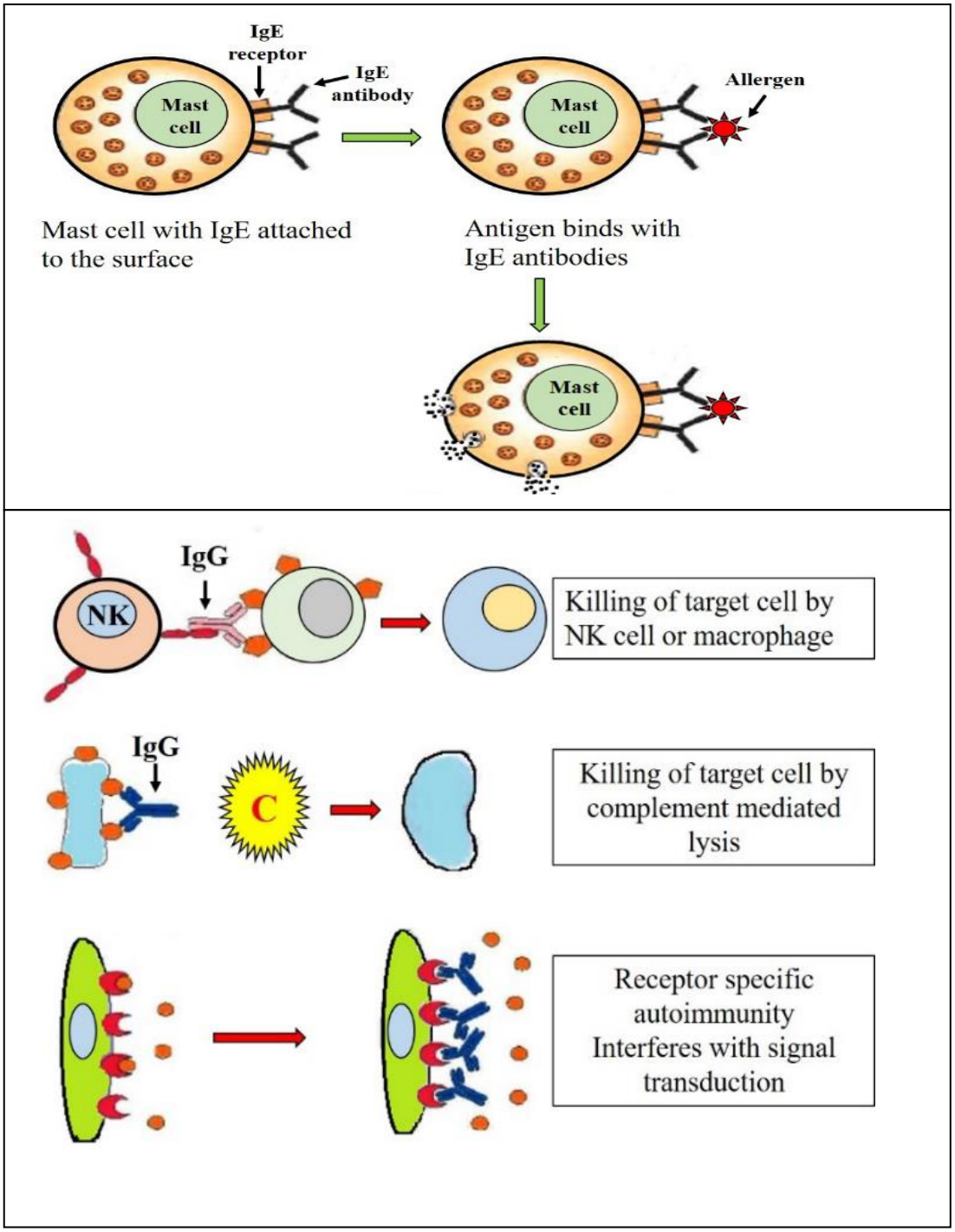
Classification of Hypersensitivity

1. TYPE I – Immediate, **A**topic, **A**naphylactic, **A**llergy (2-30 Minutes)
2. TYPE II – ANTI**B**ODY Dependant (5-8 hrs)
3. TYPE III – Immune **C**OMPLEX (2-8 hrs)
4. TYPE IV – CELL MEDIATED / **D**elayed Type of Hypersensitivity (24 hours to days)

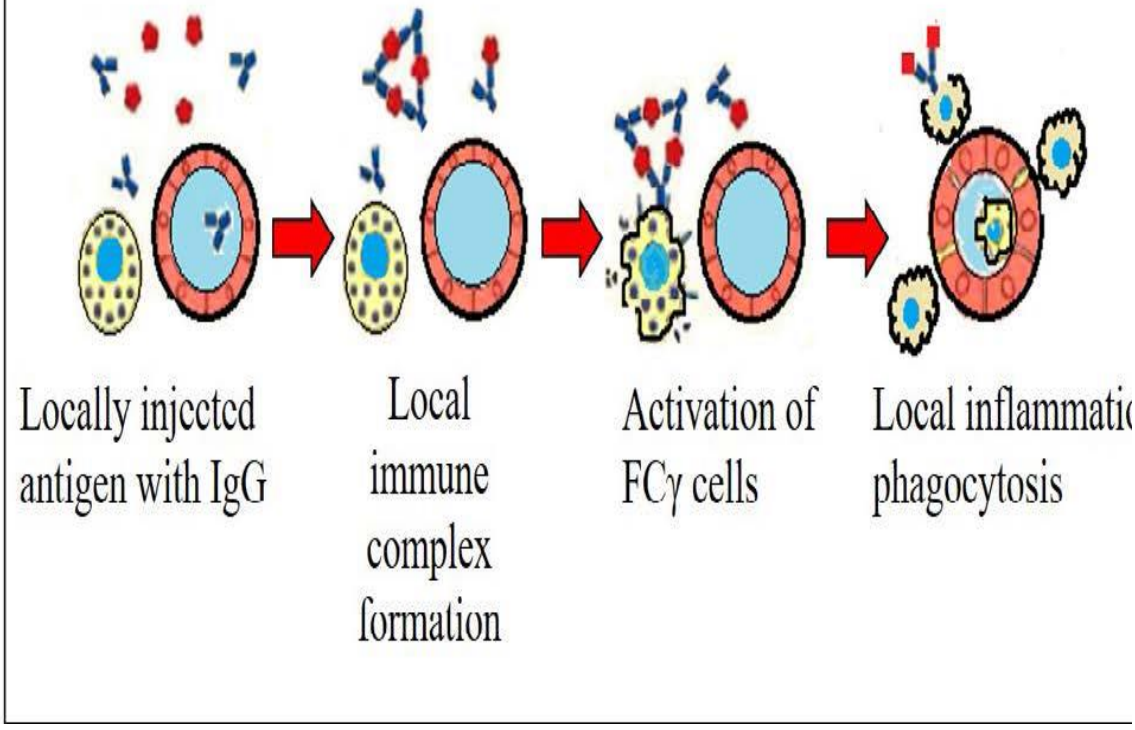
	Immune reactant	**Mechanism of activation**	**Ag form**	**Clinical syndrome**
TYPE I	IgE mediated HS	Ag induces cross linking of IgE bound to mast cells and basophils with release of vasoactive mediators	Soluble Ag	**A**topic, **A**naphylactic, **A**llergy, Hay fever, Asthma, Food allergies

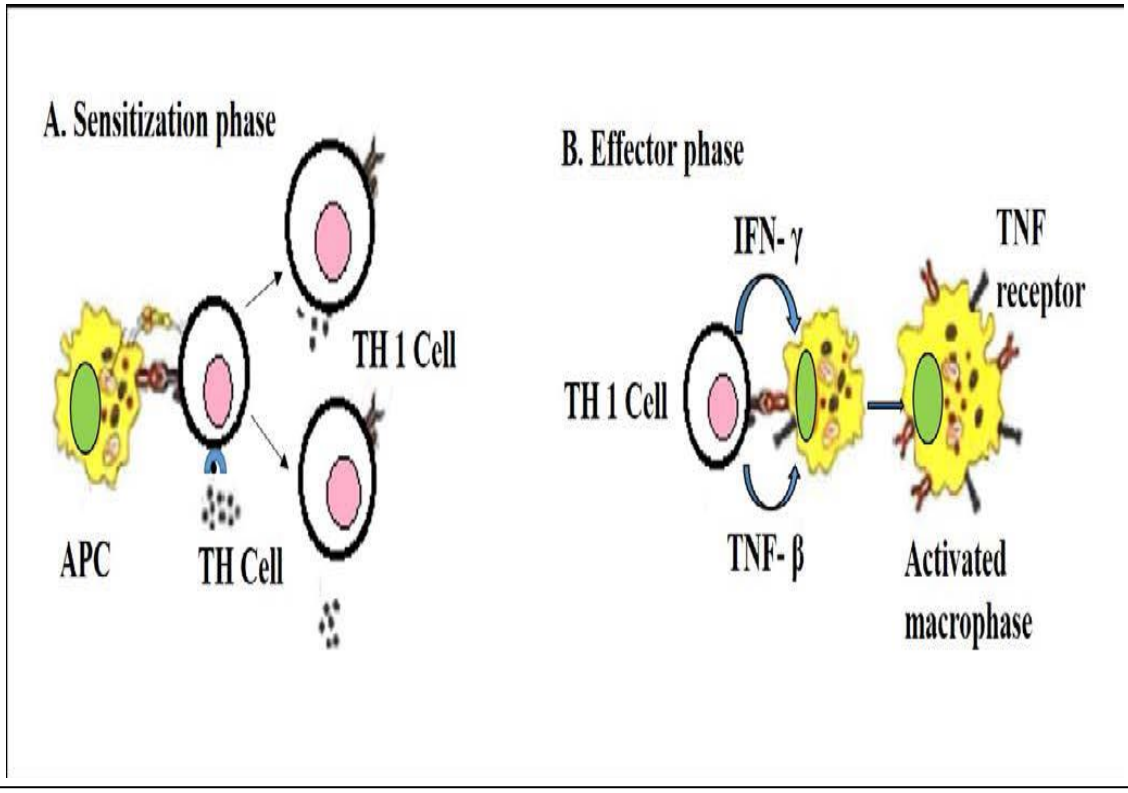
TYPE II	IgG cytotoxic HS IgG or IgM	Ab directed against cells surface antigens mediated cell destruction via complement activation	Cell bound Ag	Anti**b**ody mediated damage, Autoimmune haemolytic anemia, agranulocytosis, Erythroblastosis fetalis, Blood transfusion reactions
TYPE III	Immune **c**omplex mediated HS IgG or IgM	Ag-Ab complexes deposited in various tissues induce complement activation and an ensuring inflammatory response mediated by massive infiltration of neutrophils	Soluble Ag	Arthus reaction, Serum sickness, Glomerulonephritis, RA, systemic lupus
TYPE IV	Cell mediated HS T cells	Sensitized TH1 cells release cytokines that activate macrophages or TC cells which mediate direct cellular damage	Soluble Ag or Cell bound Ag	Tubercular lesions Contact dermatitis

Type I

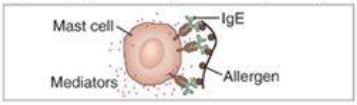
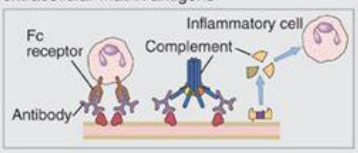
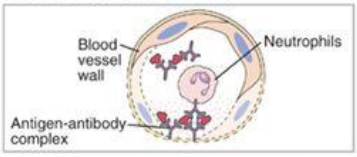
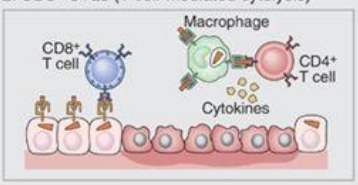

IgE receptor
IgE antibody
Mast cell
Allergen
Mast cell with IgE attached to the surface
Antigen binds with IgE antibodies
Mast cell
IgG
NK
Killing of target cell by NK cell or macrophage
IgG
C
Killing of target cell by complement mediated lysis
Receptor specific autoimmunity
Interferes with signal transduction

TYPE III


Locally injected antigen with IgG
Local immune complex formation
Activation of FCγ cells
Local inflammatic phagocytosis


A. Sensitization phase
TH 1 Cell
APC
TH Cell
B. Effector phase
IFN- γ
TNF receptor
TH 1 Cell
TNF- β
Activated macrophase

Types of Hypersensitivity Reactions

	Type of hypersensitivity	Pathologic immune mechanisms	Mechanisms of tissue injury and disease	
Type I	Immediate hypersensitivity (Type I)	T_H2 cells, IgE antibody, mast cells, eosinophils 	Mast cell-derived mediators (vasoactive amines, lipid mediators, cytokines) Cytokine-mediated inflammation (eosinophils, neutrophils)	Antibody mediated
Type II	Antibody-mediated diseases (Type II)	IgM, IgG antibodies against cell surface or extracellular matrix antigens 	Complement and Fc receptor-mediated recruitment and activation of leukocytes (neutrophils, macrophages) Opsonization and phagocytosis of cells Abnormalities in cellular function, e.g. hormone receptor signaling	
Type III	Immune complex-mediated diseases (Type III)	Immune complexes of circulating antigens and IgM or IgG antibodies deposited in vascular basement membrane 	Complement and Fc receptor-mediated recruitment and activation of leukocytes	
Type IV	T cell-mediated diseases (Type IV)	1. $CD4^+$ T cells (delayed type hypersensitivity) 2. $CD8^+$ CTLs (T cell-mediated cytolysis) 	1. Macrophage activation, cytokine-mediated inflammation 2. Direct target cell lysis, cytokine-mediated inflammation	T cell mediated

SECTION 14 - PRINCIPLES OF IMMUNOLOGY

TABLE OF CONTENTS

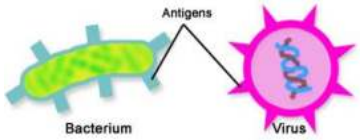
1.0 THE IMMUNE SYSTEM

1.1 INTRODUCTION

The body is protected from infectious agents and other harmful substances by a variety of cells and molecules that make up the immune system. Immunity is the ability of the human body to tolerate the presence of material indigenous to the body (self), and to eliminate foreign (non-self) material.

Foreign substances such as viruses, bacteria, toxins, and parasites are surrounded by *antigens* that, when introduced into the body, are capable of inducing a response by the immune system. This discriminatory ability provides protection from infectious disease, since most agents or associated toxins are identified as foreign by the immune system.

Figure 1 - Antigens



1.2 CELLS OF THE IMMUNE SYSTEM

The immune system depends upon the activities of three categories of white blood cells (WBCs) that are derived from bone marrow:

- Phagocytic cells:

  Macrophages and dendritic cells are phagocytic cells that reside in the blood and tissues waiting to engulf foreign substances.

- T cells:

  After leaving the bone marrow, some WBCs reach the thymus gland where they differentiate and become thymus-derived lymphocytes or T cells.

- B cells:

  WBCs that do not reach the thymus gland become B lymphocytes or B cells

1.3 LYMPHATIC SYSTEM

Some of the WBCs migrate to guard peripheral tissues, some reside within the tissues, and others circulate in the blood stream and in a specialized system of vessels and nodules in the lymphatic system.

The lymphatic system drains extracellular fluid and frees cells from tissues. The extracellular fluid and cells are transported through the body via the lymphatic vessels as lymph, and eventually emptied back into the blood system.

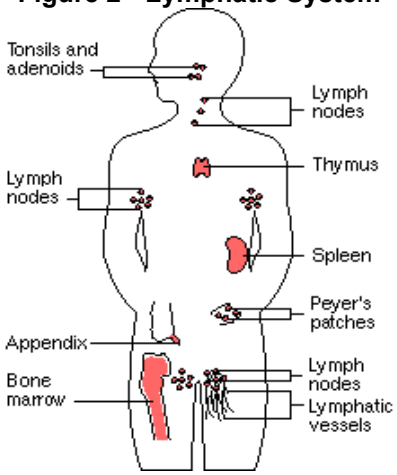
The lymphatic vessels closely parallel the body's veins and arteries. Lymph nodes are found throughout the lymphatic vessels and provide meeting areas for interaction between the immune system cells.

The lymphatic system contains the following:

Primary lymphoid organs
- Bone marrow
- Thymus
- Lymphatic vessels

Secondary lymphoid organs
- Spleen
- Lymph nodes

Figure 2 - Lymphatic System



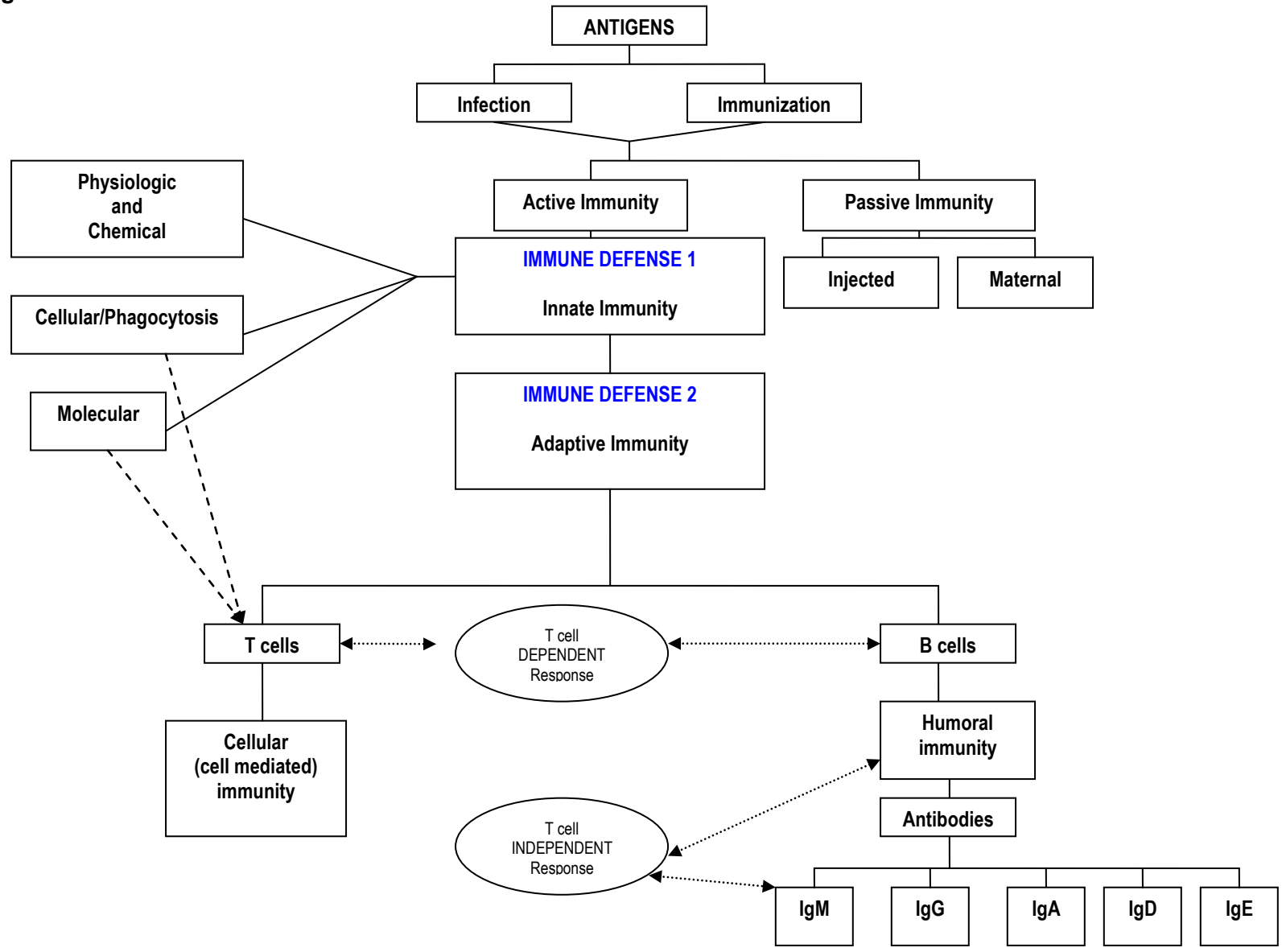
1.4 TYPES OF IMMUNITY (SEE FIGURE 3)

Passive Immunity:
- Antibodies from another person or animal that can be injected or transfused.
- Called passive because the individual did not create the antibodies, but instead received pre-formed antibodies.
- Protection is effective, but duration is short lived and no memory is created.
- Examples of passive immunity are maternal antibodies (trans-placental and breast milk) and injected antibodies (e.g., rabies, varicella, and tetanus immune globulins).

Active Immunity:

When the body is exposed to a foreign substance the cells of the immune system "actively" respond. Active immunity is further divided into categories:
- Innate Immunity - protective mechanisms we are born with
- Adaptive Immunity – cell mediated immunity and humoral immunity

Figure 3 - Immune Defenses



1.5 ACTIVE IMMUNITY

The body's immune response can be understood by following the course of an infection as described below:

- Most pathogens are kept outside of the body by protective mechanisms such as tears or skin that act as barriers.
- When there is an injury to tissue, bacteria or viruses can enter the tissue and cause infection.
- Innate cells (macrophages, dendritic cells) respond by recognizing viruses and bacteria as foreign and specialize in engulfing these invaders (*phagocytosis*). These innate cells and protective barriers are part of innate immunity because they "innately" respond to foreign substances.
- In addition, dendritic cells display the antigens on their cell surface and travel to the lymph nodes.
- In the lymph nodes the dendritic cells present the antigen to the T cells. The T cells then activate the B cells to make antibodies. The T cells and B cells are part of the adaptive immunity because they are "adapting" to the foreign substance and creating memory against future infections.

1.6 INNATE IMMUNITY - "FIRST" IMMUNE DEFENCE

Innate immunity consists of protective mechanisms we are born with, and are the first line of defence against anything recognized as non-self.

The produced immune response is not specific to the antigen and no memory of the antigen persists.

However, innate immunity is the crucial first step in most adaptive immune responses.

The following are the protective mechanisms of innate immunity (see Table 1):

- Physical and Chemical Mechanisms
- Phagocytosis
- Molecular Response
- Inflammatory Response

Table 1 - Innate Immunity			
Physical and Chemical Mechanisms	**Phagocytosis**	**Molecular Response**	**Inflammatory Response**
Physical barriers: • intact skin • mucous membrane barrier (sneezing, coughing) • cilia **Chemical barriers:** • tears • acid (pH) • saliva • bile	**Macrophages:** • engulf and kill invading organisms **Dendritic cells:** • engulf pathogen • display antigen on cell surface • travel to lymph node to present antigen to T cells • critical link between the innate and adaptive immune responses.	**Cytokines:** Cytokines are small proteins made by a cell that affect the behavior of other cells. Examples: • Cytokines cause vasodilation (heat and redness) • Some types of *interferon* are antiviral cytokines which help healthy cells resist viral infection **Chemokines:** Chemokines are proteins secreted by macrophages that attract cells out of the blood stream and into the infected tissues. **Complement:** The complement system is a group of approximately 20 proteins that coat bacterial surfaces and promote bacterial destruction by macrophages.	The accumulation of fluid and cells at the site of infection causes the redness, swelling, heat, and pain known as inflammation. Inflammation is beneficial because it: • recruits cells out of the blood stream, • increases the flow of lymph to take away microbes and antigen-bearing cells to the lymphoid tissue which will lead to adaptive immunity, and • brings the T cells and B cells back to the site of infection.

1.7 ADAPTIVE IMMUNITY - "SECOND" IMMUNE DEFENCE

Adaptive immunity is the second line of defence against anything recognized as non-self and it provides protection against re-exposure to the same pathogen.

Characteristics of adaptive immunity:

- Specificity: the immune response is specific to the antigen that produced it (e.g. antibody for measles antigen has no effect on rubella antigen)
- Tolerance: the immune response is able to differentiate between self and non-self so that body tissues are not destroyed
- Memory: with subsequent exposure to an antigen there is a rapid and strong immune response. This is called an anamnestic response.

Adaptive immunity is divided into two categories:

- Cell mediated immunity
- Humoral (antibody) immunity

1.8 ADAPTIVE IMMUNITY - CELL MEDIATED IMMUNITY

Cell mediated immunity describes any immune response where T cells have the main role.

B cells are not activated by most antigens without "help" from helper T cells. The activation of T cells is an essential first stage in virtually all adaptive immune responses. This is called the "T cell-dependent immune response".

T cells do not recognize microorganisms in the extracellular fluids. Instead, T cell receptors bind to fragments of antigens (*epitopes*) that are presented on the surface of antigen presenting cells (APC).

There are three main types of APC:

- Macrophages
- Dendritic cells
- Naïve B cells

When T cells recognize an antigen presented by the APC, they can differentiate into several different types of T cells:

- Cytotoxic T cells:

  Kill cells infected with intracellular pathogens such as viruses

- Helper T cells:
  - Activate antigen and stimulate B cells to differentiate and produce antibodies
  - Activate macrophages to become more efficient at killing the pathogen
  - Control intracellular bacterial infections (e.g. tuberculosis) that grow in intracellular membrane-bound vesicles of macrophages. The macrophages can't kill the bacteria but instead display the bacterial antigen on the surface so that it can be recognized by T cells
- Regulatory T cells:

  Suppress lymphocytes and control the immune response

1.9 ADAPTIVE IMMUNITY - HUMORAL IMMUNITY

Humoral immunity is mediated by B cells. B cells react against foreign substances in the extracellular spaces of the body by producing and secreting antibodies (Abs). These Abs are present in the biological fluids of the body (the humours); hence the term humoral immunity.

Many microorganisms multiply in the extracellular spaces of the body, and most intracellular pathogens spread by moving from cell to cell through the extracellular fluids.

These extracellular spaces are protected by humoral immunity where antibodies either kill the extracellular organism and the intracellular organism as it is moving from cell to cell or bind the pathogen and present it to T cells.

B cells display immunoglobulin molecules (antibodies) on their surface membranes, which act as receptors for the antigens. B cell antibody receptors can either bind to helper T cells that have interacted with an APC or bind to extracellular microorganisms such as bacteria.

Once an antigen binds to an antibody with the best "fit", the B cell differentiates into plasma cells or B memory cells.

- Plasma cells:

  These cells operate as factories to manufacture the chosen antibody and then secrete those antibodies.
- B memory cells:

  These cells mediate immunological memory. They respond rapidly on re-exposure to the antigen that originally induced them.

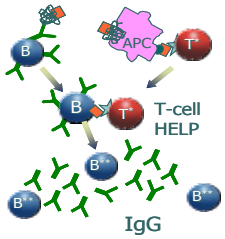
Except for the phenomenon of cross-protective immunity, each antibody can recognize and bind to only one specific antigen and no other.

Seroconversion is the phase of an infection when antibodies against an infecting agent are first detectable in the blood. To test for immunity against a particular disease an antibody titre may be ordered to assess the amount of circulating antibody specific to that pathogen.

T cell-dependent antigens (Figure 4)

- Most antigens require the interaction of T cells and B cells to generate the production of antibodies. These antigens are referred to as T cell-dependent antigens.
- The antibodies produced in response to T cell-dependent antigens are primarily IgG and the response produces immunologic memory.

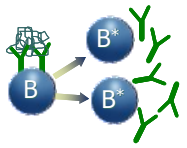
Figure 4
T- cell Dependent



T cell-independent antigens (Figure 5)

- In some situations, B cells can create antibodies without the help of T cells.
- Many common extracellular bacteria (e.g. *Haemophilus influenzae* type b) are surrounded by a polysaccharide capsule that enables them to resist ingestion by phagocytes and therefore avoid stimulating the T cell response
- Antibodies produced are of the IgM class and immunologic memory is not created.

Figure 5
T-cell Independent



1.10 ANTIBODIES

Classes of Antibodies

There are five classes of antibodies: IgM, IgG, IgA, IgD and IgE. Each class performs particular functions. The immune response to injected vaccines involves IgG and IgM. Antibodies as a class are known as immunoglobulins:

Immunoglobulin M (IgM):

- A valuable diagnostic marker for infectious disease because it is usually the first immunoglobulin made following Ag exposure and is relatively short-lived
- Effective in activating complement
- Participates in the lysis (bursting apart) of cells
- Generally remains in the blood; does not diffuse into the surrounding tissues due to its large size

Immunoglobulin G (IgG):

- The most abundant class of antibody, constituting approximately 80% of all antibodies in serum
- Produced slowly upon primary exposure to an antigen
- Produced rapidly during secondary or subsequent exposure, becoming the major antibody present
- The principal humoral component of immunological memory
- The only antibody that crosses the placenta. It helps protect the newborn from infection through passive immunity.

Immunoglobulin A (IgA):

- Represents approximately 10% to 20% of the immunoglobulins in serum
- Most abundant immunoglobulin in tissues
- Prevents or interferes with the attachment of viruses and bacteria to mucosa of respiratory and digestive systems
- Protects against enterotoxins released by certain bacteria; for example, forms an antibody-antigen complex with cholera toxin, preventing it from binding to specific receptors on the intestinal membrane
- Plays a role in eliminating food antigens from the circulatory system
- The main secretory immunoglobulin; found in exocrine secretions (e.g., breast milk, saliva, tears, respiratory and digestive secretions, urine).

Immunoglobulin D (IgD)

- Constitutes only a very small fraction (0.2%) of immunoglobulin in the body
- Acts as an antigen receptor on the surfaces of B cells
- Unknown activity.

Immunoglobulin E (IgE)

- Minute concentration in serum
- Involved in mediating allergic reactions
- Elevated in people with hypersensitivity to allergens, as well as those with eczema, asthma, or other respiratory problems
- Especially useful against parasitic infections (e.g., worms)

Antibody function

Antibodies have three main functions:

1) Neutralization:
   Antibodies bind to pathogens (e.g., viruses, bacteria) and block their access to cells, then the antibody-antigen complex is engulfed by a macrophage.

2) Opsonization:
Encapsulated bacteria (e.g., Hib, pnemococcal, and meningococcal) evade the innate immune system because they are not recognized by macrophages or dendritic cells. However, encapsulated bacteria can be recognized by antibodies. The antibody coats the bacteria to enable the ingestion by macrophages and dendritic cells through the process of opsonization.

3) Complement Activation:
Antibodies bind to certain bacteria in the plasma. A region on the antibody is a receptor to complement proteins which will help lyse the bacteria or attract the macrophages to it.

Figure 6 - Antibody Function

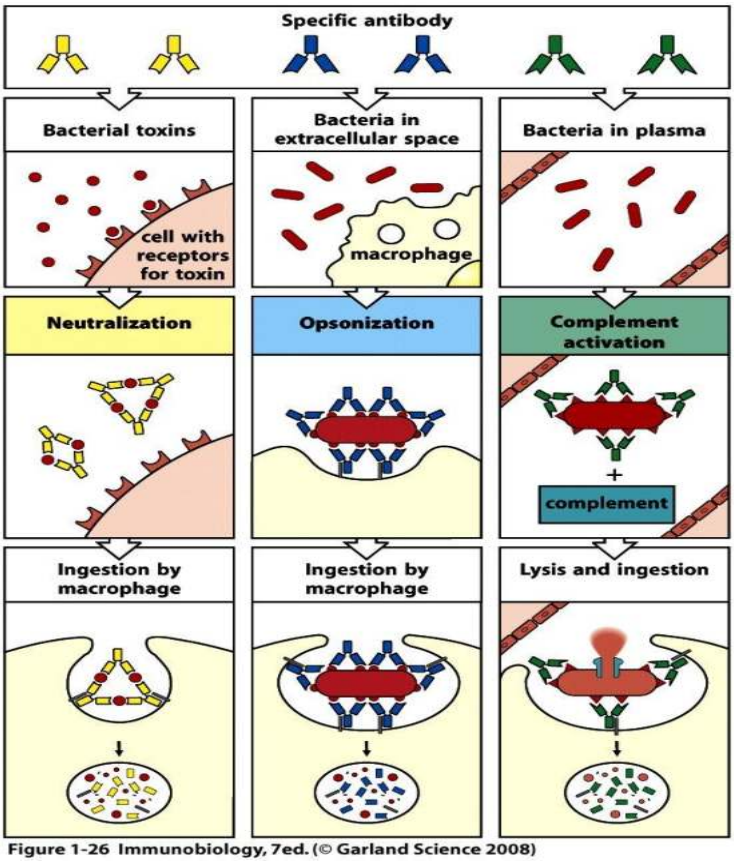


Figure 1-26 Immunobiology, 7ed. (© Garland Science 2008)

Figure 6: ©2007 From Janeway's Immunobiology, 6E by Murphy et al. Reproduced by permission of Garland Science/Taylor & Francis, LLC.

1.11 FETAL AND INFANT IMMUNE SYSTEM

The development of the immune system occurs early in fetal development.

Table 2 - Fetal Immunity	
Passive Immunity (passage of maternal antibody -IgG only)	**Active Immunity**

• Beginning at 8 weeks gestational age (GA)
• IgG levels correlate with GA:
  • low until 20 weeks
  • by 40 weeks, it doubles that of 32 wks
	• B cells and T cells present by 14 weeks GA
	• Relatively sterile environment in utero. There is an enormous unchallenged capacity

Newborns (even premature infants) can actively distinguish self from non-self.

Table 3 - Neonatal and Infant Immunity		
Birth	**Passive Immunity** (maternally acquired antibodies)	**Active Immunity**
• Instant challenge • Within hours, GI tract heavily colonized	• Circulating placental IgG lasts 6 months or longer • Secretory IgA in breast milk and colostrum	• B cell responses are good. Until two years of age, children do not respond well to T cell-independent antigens (i.e., polysaccharides) • Full T cell subsets. Infants respond well to T cell-dependent antigens (i.e., proteins)

2.0 IMMUNIZING AGENTS

Immunizing agents are classified as passive or active.

2.1 PASSIVE IMMUNIZING AGENTS

The prevention of illness through the transfer of pre-formed IgG antibodies is called immunoprophylaxis.

While the protection is immediate, it is temporary and it can only be offered if the exposure is recognized.

Protection is also time-sensitive. Post-exposure immunoprophylaxis must be initiated within a short time frame, usually within days of exposure to the infection.

Passive agents may not be completely free of blood borne pathogens despite all current safeguards and technology in place.

Types of Passive Immunizing Agents:

- Standard immune globulin – pooled antibody from thousands of donors. It is now primarily used for post-exposure prophylaxis against measles.
- Hyperimmune globulins – made from donated plasma of persons with high levels of a specific IG (e.g., Hepatitis B Immune Globulin).
- Hyperimmune serum – produced in animals (e.g., botulinum and diphtheria antitoxins).

Table 4 - Passive Immunizing Agents		
Agent		**Indication/Action**
Standard Immune Globulin	Immune globulin (human)	Exposure to measles for susceptible individuals who cannot receive live attenuated measles-containing vaccine; exposure to Hepatitis A for individuals who cannot receive Hepatitis A vaccine
Hyperimmune Globulins	Varicella-zoster immune globulin (VarIg)	VarIg is recommended for high risk susceptible people with significant exposure to varicella
	Rabies immune globulin (RabIg)	Post-exposure prophylaxis against rabies in susceptible exposed individuals
	Tetanus immune globulin (TIg)	TIg neutralizes tetanus toxin in the body fluids
	Hepatitis B immune globulin (HBIg)	Provides immediate and effective short-term passive immunity to hepatitis B
Hyperimmune Serum	Botulism antitoxin (equine)	Recommended for established or suspected botulism
	Diphtheria antitoxin (equine)	Neutralizes diphtheria toxin in the body fluids

2.2 ACTIVE IMMUNIZING AGENTS

Protection acquired through active immunizing agents is produced by one's own immune response. Protection takes longer than with passive immunizing agents, but is stronger and may be permanent.

Table 5 – Active Immunizing Agents		
Replicating Vaccines	Virus	MMR, Varicella, Yellow Fever
	Bacteria	Typhoid (oral)
Non-Replicating Vaccines	Viral	Polio (inj), hepatitis A, rabies, JEV
	Bacteria	Typhoid (inj)
	Proteins (subunit)	Acellular pertussis, influenza
	Protein toxoid (subunit)	Diphtheria, tetanus
	Recombinant (subunit)	Hepatitis B, Human papillomavirus
	Polysaccharide (subunit)	Pneumococcal, Meningococcal, Typhoid (inj)
	Conjugate (subunit)	Act-HIB, Meningococcal, Pneumococcal

2.2.1 Replicating Vaccines - Live Attenuated Vaccines

These vaccines contain whole, living virus or bacteria that induce immunity by actively replicating within the host.

Attenuated means the vaccine strains are weakened so that infection is usually unapparent or very mild.

Because these vaccines replicate, the immune response is both cell mediated and humoral and therefore protection is long-lasting, probably life-long.

Limitations:

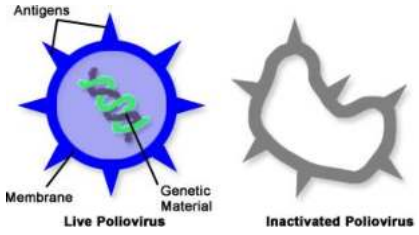
- Circulating antibodies can interfere with vaccine virus replication
- Sensitive to exposure to heat and light
- Use with caution/contraindicated in immunocompromised persons
- Live vaccines must be given on the same day or 28 days apart because circulating interferon may interfere with the replication of the second live vaccine.

2.2.2 Non-Replicating Vaccines

Because these vaccines do not replicate, protection takes longer to achieve as more vaccine doses are needed to create a protective immune response. After the scheduled numbers of vaccine doses are given, the immune response is strong and may be permanent. With some vaccines, antibody levels may fall over time and as a result booster doses may be needed.

- Inactivated Vaccines (Figure 7):
  Inactivated vaccines contain killed bacteria or viruses.

Figure 7 - Inactivated Vaccines



- Subunit Vaccines
  Subunit vaccines contain purified products that usually come from the bacteria or virus that causes natural infection, but may also be synthesized in the laboratory using recombinant technology.

  - Proteins
    Purified, inactivated proteins from the outer coating of viruses or bacteria. Aluminum salt is added as an adjuvant to enhance the immune response.

  - Protein Toxoid
    Vaccines made from inactivated bacterial toxins. Aluminum salt is added as an adjuvant to enhance the immune response.

- Recombinant Vaccines
  Vaccine antigens produced using genetic engineering technology.

- Polysaccharide Vaccines
  Polysaccharide vaccines are composed of long chains of sugar molecules that make up the surface capsule of encapsulated bacteria. The immune response to a pure polysaccharide vaccine is typically T cell-independent.

- Conjugate Vaccines
  By linking a polysaccharide to a protein (diphtheria toxoid protein is commonly used) the immune response becomes T cell-dependent and immunogenicity is improved in infants and young children < 2 years of age. This process is called conjugation; hence the term “conjugate vaccines.”

3.0 VACCINE IMMUNE RESPONSE

3.1 INTRODUCTION

Vaccines interact with the immune system and produce an immune response similar to that produced by the natural infection, but they do not subject the recipient to the disease and its potential complications. Vaccines produce immunological memory similar to that acquired by having the natural disease. The antigen is the part of the vaccine that stimulates the immune response.

The immune or antibody response to non-replicating vaccines (inactivated/subunit) is different from the response to replicating vaccines (live attenuated).

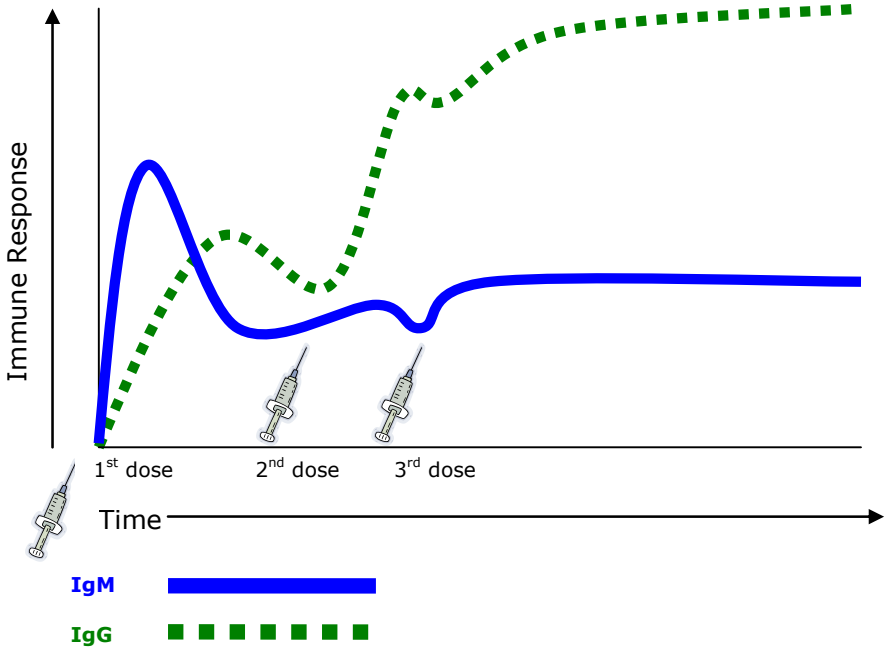
3.2 ANTIBODY RESPONSE TO A NON-REPLICATING VACCINE

Inactivated/subunit vaccines will need more doses to build an adequate and lasting immune response (Figure 8).

- Primary immune response:
  Antibody following the first exposure to an antigen is primarily IgM. Response is of brief duration and low intensity.

- Secondary immune response:
  Antibody following the second and subsequent immunogenic challenges is primarily IgG. Memory cells are already present at time of repeat exposure and make the specific antibodies more rapidly.

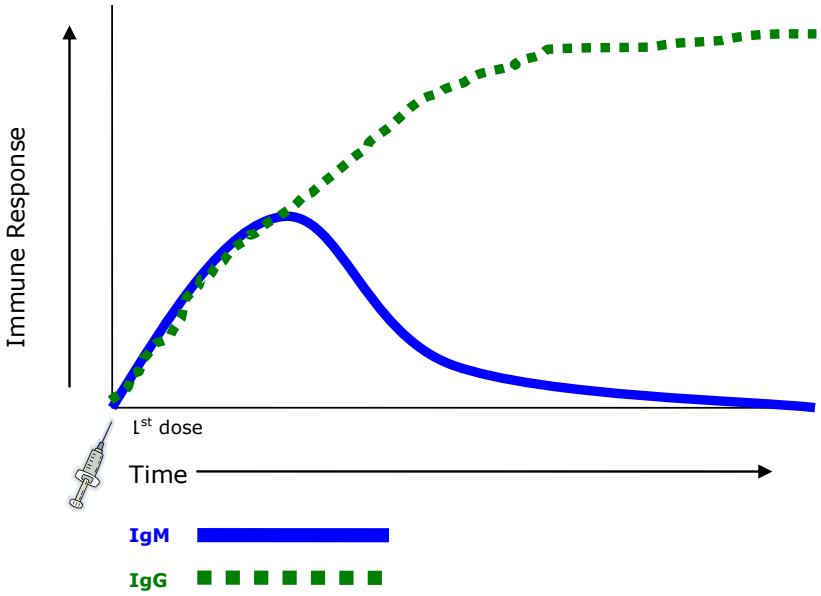
Figure 8 - Immune Response to Non-Replicating Vaccines



3.3 ANTIBODY RESPONSE TO A REPLICATING VACCINE

The immune response to a live attenuated vaccine is virtually identical to that produced by a natural infection as cell-mediated and humoral immunity are fully activated. Replicating vaccines will need fewer doses to create lasting memory.

Figure 9 - Immune Response to Replicating Vaccines
Live Attenuated Vaccines

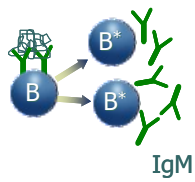


3.4 ANTIBODY RESPONSE TO CONJUGATE AND POLYSACCHARIDE VACCINES

Polysaccharide Vaccine Immune Response (Figure 10)

- Polysaccharide vaccines stimulate B cells without the help of T cells, resulting in a T cell-independent immune response
- The antibody made in response to these vaccines is mostly of the IgM class and immunologic memory is not produced.
- Polysaccharide vaccines are not immunogenic in children < 2 years of age.

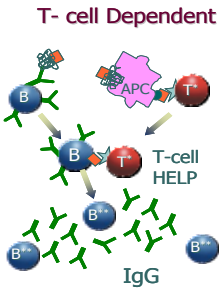
Figure 10 - Polysaccharide Vaccine Immune Response



Conjugate Vaccine Immune Response (Figure 11)

- Conjugate vaccines stimulate T cells and B cells, resulting in a T cell-dependent immune response
- The antibodies produced include IgG, providing longer protection and immunologic memory.
- Conjugate vaccines are immunogenic in children < 2 years of age

Figure 11 - Conjugate Vaccine Immune Response



3.5 FACTORS THAT INFLUENCE THE VACCINE IMMUNE RESPONSE:

Vaccine-related factors:

- Nature of the antigen

  Live, inactivated/subunit, conjugate and polysaccharide vaccines each generates immune responses of a different intensity and duration.

- Dose of the antigen

  A certain threshold dose of antigen is required to elicit an immune response.

- Presence of vaccine adjuvants

  Vaccine adjuvants are added to the inactivated and subunit vaccines to enhance the immune response through three mechanisms: the “depot effect” increases the immunologic half-life of the antigen at the injection site; adjuvants induce the production of cytokines to assist T cells; and adjuvants induce dendritic cell maturation.

Host-related factors:

- Circulating antibodies:

  Antibody from any source (e.g., maternal, transfusion) can interfere with live vaccine replication. For example: potential antibody interference is the reason to defer MMR vaccine until 1 year of age when maternal antibodies have declined. Administration of immune globulins (Ig) may interfere with live vaccine replication.

- Age of the client:

  Protection should precede the age of greatest risk (e.g., human papillomavirus vaccine is ideally given before an individual is sexually active). Children <2 years do not mount a protective immune response to T cell-independent antigens such as polysaccharides. Conjugate vaccines will produce an immune response in young children.

- Genetics:

  Deficiencies in the terminal components of complement and properdin result in an impaired immune response.

- Nutritional factors:

  Malnutrition results in reduction of cell-mediated immunity.

- Co-existing disease: e.g., immunodeficiency.

- Previous exposure to antigen/vaccine (anamnestic response)

3.6 VACCINE ANTIGEN LOAD

3.6.1 Capacity of the Immune System

The immune system has the capacity to respond to extremely large numbers of antigens:

- There are 10^9 to 10^{11} different antibody specificities
- 2 billion T helper cells are replenished each day

Each infant has the theoretical capacity to respond to about 10,000 vaccines at any one time. Using this estimate, if 11 vaccines were given to an infant at one time, then about 0.1% of the immune system is needed to respond.

3.6.2 Vaccine Antigen Load: "Then and Now"

Even though the number of vaccines given to a child has increased over the past 45 years, the number of antigens has decreased. There are fewer antigens per vaccine because of:

- improved purification processes;
- knowledge of antigens needed to induce protective immunity.

Figure 12 - Vaccine antigen load. Source: Offit, P.A., Quarles et al (2002). Addressing parents' concerns: Do multiple vaccines overwhelm or weaken the infants' immune system? *Paediatrics*. Vol 109, pages 124 - 129.

TABLE 2. Number of Immunogenic Proteins and Polysaccharides Contained in Vaccines Over the Past 100 Years

1900		1960		1980		2000	
Vaccine	Proteins	Vaccine	Proteins	Vaccine	Proteins	Vaccine	Proteins/ Polysaccharides
Smallpox*	~200	Smallpox	~200	Diphtheria	1	Diphtheria	1
Total	**~200**	Diphtheria†	1	Tetanus	1	Tetanus	1
		Tetanus‡	1	WC-Pertussis	~3000	AC-Pertussis¶¶	2–5
		WC-Pertussis§	~3000	Polio	15	Polio	15
		Polio‖	15	Measles¶	10	Measles	10
		Total	**~3217**	Mumps#	9	Mumps	9
				Rubella**	5	Rubella	5
				Total	**~3041**	Hib††	2
						Varicella‡‡	69
						Pneumococcus§§	8
						Hepatitis B‖‖	1
						Total	**123–126**

* *Vaccinia vaccine*: Goebel SJ, Johnson GP, Perkus ME, et al. *Virology*. 1990;179:247–266.
† *Diphtheria toxoid*: *MMWR Morb Mortal Wkly Rep*. 1991 (August);40:1–28.
‡ *Tetanus toxoid*: *MMWR Morb Mortal Wkly Rep*. 1991 (August);40:1–28.
§ *Whole cell pertussis vaccine*: Number estimated from genome size. The sequence of Bordetella pertussis Tohama I strain will soon be completed at the Sanger Center in Great Britain.
‖ *Polio vaccine*: Wimmer E, Nomoto A. *Biologicals*. 1993;21:349–356; Kitamura N, Semler BL, Rothberg PG, et al. *Nature*. 1981;291:547–553; Five proteins per poliovirus virion and 3 poliovirus strains in the inactivated poliovirus vaccine (IPV).
¶ *Measles vaccine*: Griffen D, Bellini WL. Measles virus. In: Fields BN, ed. Knipe DM, Howley PM, et al, eds. Philadelphia, PA: Lipincott-Raven Publishers; 1996.
# *Mumps vaccine*: Elango N, Varsanyi TM, Kovamees J, Norrby E. *J Gen Virol*. 1988;69:2893–2900.
** *Rubella vaccine*. Hofmann J, Gerstenberger S, Lachmann I, et al. *Virus Res*. 2000;68:155–160.
†† *Conjugate Haemophilus influenzae type b vaccine*: *MMWR Morb Mortal Wkly Rep*. 1991 (January);40:1–7.
‡‡ *Varicella vaccine*: Cohen JI. *Infect Dis Clin North Am*. 1996;10:457–468.
§§ *Streptococcus pneumoniae vaccine*: *MMWR Morb Mortal Wkly Rep*. 2000;49:1–29.
‖‖ *Hepatitis B vaccine*: *MMWR Morb Mortal Wkly Rep*. 1991 (November);40:1–25.
¶¶ *Acellular pertussis vaccine*: *MMWR Morb Mortal Wkly Rep*. 1997 (March);46:1–25.

4.0 GLOSSARY

Active immunity
When the body is exposed to a foreign substance the cells of the immune system "actively" respond

Adaptive immunity
The cells of the immune system "adapt" to the foreign substance and create memory against future infections.

Adjuvant
A substance added to a vaccine to enhance the immune response by intensity and/or duration.

Anamnestic response
A renewed rapid production of an antibody on the second (or subsequent) encounter with the same antigen

Antigen
A foreign substance which, when introduced into the body is capable of inducing an immune response.

APC (antigen presenting cell)
Highly specialized cells that process antigens and display their peptide fragments on their surface

Cytotoxic T cells
Kill intracellular pathogens like viruses

Dendritic cells
Phagocytic cells found in most tissues. They act as antigen presenting cells (APC), travel to lymph nodes and present antigens to T cells

Epitope
The site on an antigen that is recognized by an antibody or an antigen receptor

Helper T cells
T cells that stimulate B cells to differentiate and produce antibodies

Immunoglobulin
A class of antibodies

Immunoprophylaxis
Disease prevention by immunologic means. Active immunoprophylaxis involves the administration of vaccines to stimulate the host's own immune system. Passive immunoprophylaxis involves the administration of immune globulins from an immune donor.

Innate immunity
Protective mechanisms we are born with e.g., cilia, skin, mucosal membranes

Interferon
Antiviral cytokines which help healthy cells resist viral infection

Lymph
Extracellular fluid that accumulates in tissues and is carried through the lymphatic system by the lymphatic vessels

Lysis
Process of disintegration or dissolution of cells

Macrophage
Large phagocytic cell; creates inflammation cytokines; acts as an antigen presenting cell (APC)

Memory cell
Lymphocytes that mediate immunological memory

Passive immunity
Antibodies from another person or animal are injected or transfused into an individual who receives "passive" immunity

Phagocytosis
The process by which a cell engulfs and digests solid substances

Plasma Cell
Specialized B cells that create antibodies

Regulatory T cells
Suppress lymphocytes and control the immune response

Seroconversion
The phase of an infection when antibodies against a pathogen are detected in the blood

5.0 REFERENCES

Centers for Disease Control and Prevention (2009). Principles of vaccination. *Epidemiology and Prevention of Vaccine -Preventable Diseases* (11th ed.). http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/prinvac.pdf.

Murphy, K., Travers, P., Walport, M., (2008). *Janeway's immunobiology* (7th ed.). Garland Science.

Offit, P.A., Quarles et al (2002). Addressing parents' concerns: Do multiple vaccines overwhelm or weaken the infants' immune system? *Paediatrics*. Vol 109, pages 124 - 129.

Public Health Agency of Canada (2006). *Canadian immunization guide* (7th ed.). http://www.phac-aspc.gc.ca/publicat/cig-gci/index-eng.php.

6.0 RESOURCES

The following documents are available on line, as well as in print, free of charge:

National Institutes of Health (2007). *Understanding the immune system. How it works*. U.S. Department of Health and Human Services. http://www3.niaid.nih.gov/topics/immuneSystem/PDF/theImmuneSystem.pdf

National Institutes of Health (2007). *Understanding vaccines. What they are and how they work.* U.S. Department of Health and Human Services. http://www3.niaid.nih.gov/topics/vaccines/PDF/undvacc.pdf

Precipitation Reactions

Antibody and soluble antigen interacting in aqueous solution form a lattice that eventually develops into a visible precipitate. Antibodies that aggregate soluble antigens are called precipitins. Although formation of the soluble Ag-Ab complex occurs within minutes, formation of the visible precipitate occurs more slowly and often takes a day or two to reachcompletion.

Formation of an Ag-Ab lattice depends on the valency of both the antibody and antigen:

- The antibody must be bivalent; a precipitate will not form with monovalent Fab fragments.
- The antigen must be either bivalent or polyvalent; that is, it must have at least two copies of the same epitope, or have different epitopes that react with different antibodies present in polyclonal antisera.

Experiments with myoglobin illustrate the requirement that protein antigens be bivalent or polyvalent for a precipitin reaction to occur. Myoglobin precipitates well with specific polyclonal antisera but fails to precipitate with a specific monoclonal antibody because it contains multiple, distinct epitopes but only a single copy of each epitope (Figure 6-4a). Myoglobin thus can form a crosslinked lattice structure with polyclonal antisera but not with monoclonal antisera. The principles that underlie precipitation reactions are presented because they are essential for an understanding of commonly used immunological assays. Although various modifications of the precipitation reaction were at one time the major types of assay used in immunology, they have been largely replaced by methods that are faster and, because they are far more sensitive, require only very small quantities of antigen or antibody. Also, these modern assay methods are not limited to antigen-antibody reactions that produce a precipitate.

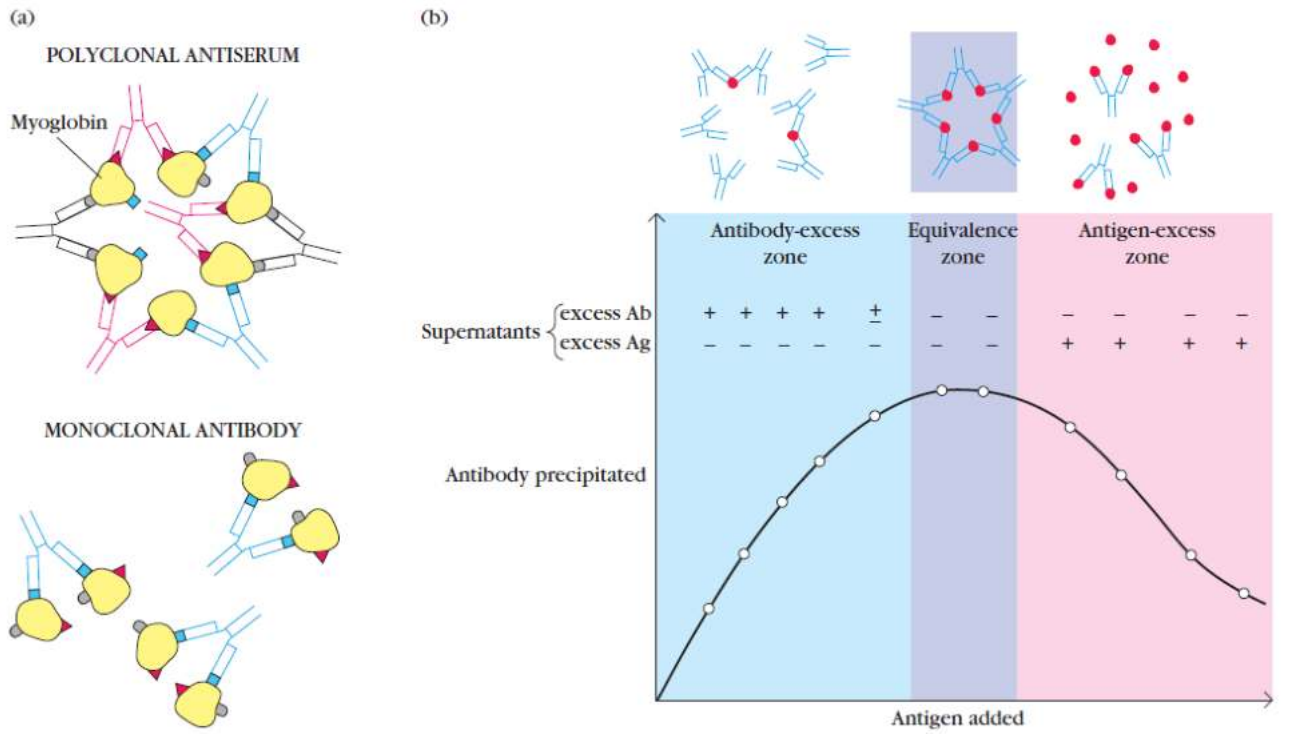


FIGURE 6-4 Precipitation reactions. (a) Polyclonal antibodies can form lattices, or large aggregates, that precipitate out of solution. However, if each antigen molecule contains only a single epitope recognized by a given monoclonal antibody, the antibody can link only two molecules of antigen and no precipitate is formed. (b) A precipitation curve for a system of one antigen and its antibodies. This plot of the amount of antibody precipitated versus increasing antigen concentrations (at constant total antibody) reveals three zones: a zone of antibody excess, in which precipitation is inhibited and antibody not bound to antigen can be detected in the supernatant; an equivalence zone of maximal precipitation in which antibody and antigen form large insoluble complexes and neither antibody nor antigen can be detected in the supernatant; and a zone of antigen excess in which precipitation is inhibited and antigen not bound to antibody can be detected in the supernatant.

Precipitation Reactions in Fluids Yield a Precipitin Curve: A quantitative precipitation reaction can be performed by placing a constant amount of antibody in a series of tubes and adding increasing amounts of antigen to the tubes. At one time this method was used to measure the amount of antigen or antibody present in a sample of interest. After the precipitate forms, each tube is centrifuged to pellet the precipitate, the supernatant is poured off, and the amount of precipitate is measured. Plotting the amount of precipitate against increasing antigen concentrations yields a precipitin curve. As Figure 6-4b shows, excess of either antibody or antigen interferes with maximal precipitation, which occurs in the so-called equivalence zone, within which the ratio of antibody to antigen is optimal. As a large multi molecular lattice is formed at equivalence, the complex increases in size and precipitates out of solution. As shown in Figure 6-4, under conditions of antibody excess or antigen excess, extensive lattices do not form and precipitation is inhibited. Although the quantitative precipitation reaction is seldom used experimentally today, the principles of antigen excess, antibody excess, and equivalence apply to many Ag-Ab reactions.

Precipitation Reactions in Gels Yield Visible Precipitin Lines: Immune precipitates can form not only in solution but also in an agar matrix. When antigen and antibody diffuse toward one another in agar, or when antibody is incorporated into the agar and antigen diffuses into the antibody-containing matrix, a visible line of precipitation will

form. As in a precipitation reaction in fluid, visible precipitation occurs in the region of equivalence, whereas no visible precipitate forms in regions of antibody or antigen excess. Two types of immunodiffusion reactions can be used to determine relative concentrations of antibodies or antigens, to compare antigens, or to determine the relative purity of an antigen preparation. They are radial immunodiffusion (the Mancini method) and double immunodiffusion (the Ouchterlony method); both are carried out in a semisolid medium such as agar. In radial immunodiffusion, an antigen sample is placed in a well and allowed to diffuse into agar containing a suitable dilution of an antiserum. As the antigen diffuses into the agar, the region of equivalence is established and a ring of precipitation, a precipitin ring, forms around the well (Figure 6-5, upper panel). The area of the precipitin ring is proportional to the concentration of antigen. By comparing the area of the precipitin ring with a standard curve (obtained by measuring the precipitin areas of known concentrations of the antigen), the concentration of the antigen sample can be determined. In the Ouchterlony method, both antigen and antibody diffuse radially from wells toward each other, thereby establishing a concentration gradient. As equivalence is reached, a visible line of precipitation, a precipitin line, forms (Figure 6-5, lower panel).

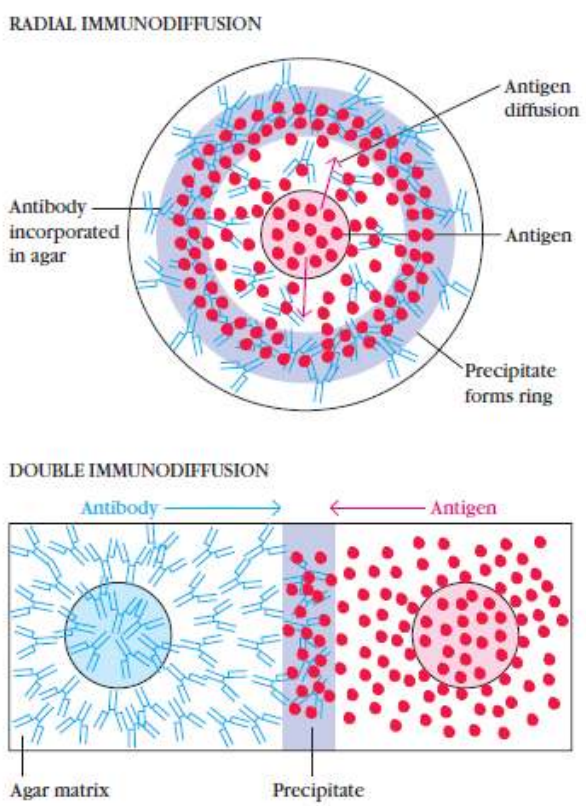


FIGURE 6-5 Diagrammatic representation of radial immunodiffusion (Mancini method) and double immunodiffusion (Ouchterlony method) in a gel. In both cases, large insoluble complexes form in the agar in the zone of equivalence, visible as lines of precipitation (purple regions). Only the antigen (red) diffuses in radial immunodiffusion, whereas both the antibody (blue) and antigen (red) diffuse in double immunodiffusion.

Immunoelectrophoresis Combines Electrophoresis and Double Immunodiffusion: In immunoelectrophoresis, the antigen mixture is first electrophoresed to separate its components by charge. Troughs are then cut into the agar gel parallel to the direction of the electric field, and antiserum is added to the troughs. Antibody and antigen then diffuse toward each other and produce lines of precipitation where they meet in appropriate proportions (Figure 6-6a). Immunoelectrophoresis is used in clinical laboratories to detect the presence or absence of proteins in the serum. A sample of serum is electrophoresed, and the individual serum components are identified with antisera specific for a given protein or immunoglobulin class. This technique is useful in determining whether a patient produces abnormally low amounts of one or more isotypes, characteristic of certain immunodeficiency diseases. It can also show whether a patient overproduces some serum protein, such as albumin, immunoglobulin, or transferrin. The immunoelectrophoretic pattern of serum from patients with multiple

myeloma, for example, shows a heavy distorted arc caused by the large amount of myeloma protein, which is monoclonal Ig and therefore uniformly charged. Because immunoelectrophoresis is a strictly qualitative technique that only detects relatively high antibody concentrations (greater than several hundred g/ml), it utility is limited to the detection of quantitative abnormalities only when the departure from normal is striking, as in immunodeficiency states and immunoproliferative disorders. A related quantitative technique, rocket electrophoresis, does permit measurement of antigen levels. In rocket electrophoresis, a negatively charged antigen is electrophoresed in a gel containing antibody. The precipitate formed between antigen and antibody has the shape of a rocket, the height of which is proportional to the concentration of antigen in the well. One limitation of rocket electrophoresis is the need for the antigen to be negatively charged for electrophoretic movement within the agar matrix. Some proteins, immunoglobulins for example, are not sufficiently charged to be quantitatively analyzed by rocket electrophoresis; nor is it possible to measure the amounts of several antigens in a mixture at the same time.

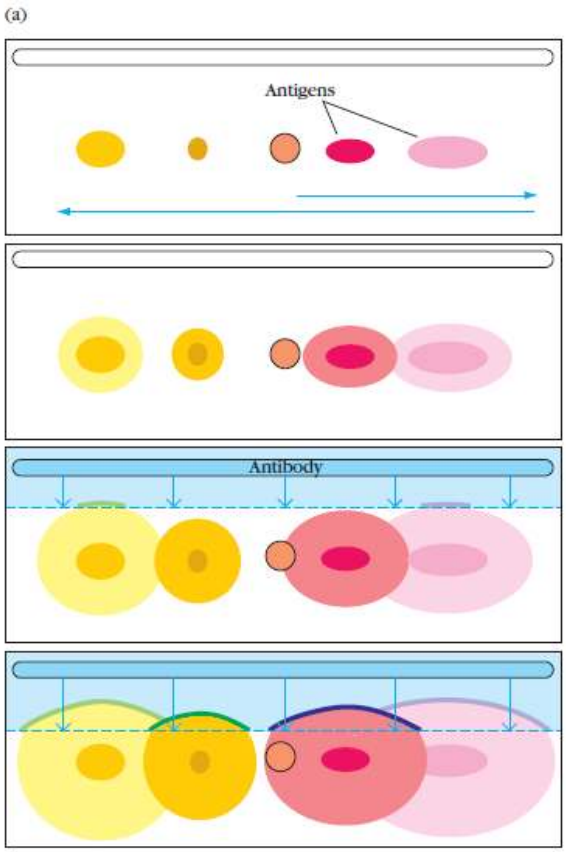


FIGURE 6-6 Immunoelectrophoresis of an antigen mixture.
(a) An antigen preparation (orange) is first electrophoresed, which separates the component antigens on the basis of charge. Antiserum (blue) is then added to troughs on one or both sides of the separated antigens and allowed to diffuse; in time, lines of precipitation (colored arcs) form where specific antibody and antigen interact.

Agglutination Reactions

The interaction between antibody and a particulate antigen results in visible clumping called agglutination. Antibodies that produce such reactions are called agglutinins. Agglutination reactions are similar in principle to precipitation reactions; they depend on the crosslinking of polyvalent antigens. Just as an excess of antibody inhibits precipitation reactions, such excess can also inhibit agglutination reactions; this inhibition is called the prozone effect. Because prozone effects can be encountered in many types of immunoassays, understanding the basis of this phenomenon is of general importance. Several mechanisms can cause the prozone effect. First, at high antibody concentrations, the number of antibody binding sites may greatly exceed the number of epitopes. As a result, most antibodies bind antigen only univalently instead of multivalently. Antibodies that bind univalently cannot crosslink one antigen to another. Prozone effects are readily diagnosed by performing the assay at a variety of antibody (or antigen) concentrations. As one dilutes to an optimum antibody concentration, one sees higher levels of agglutination or whatever parameter is measured in the assay being used. When one is using polyclonal antibodies, the prozone effect can also occur for another reason. The antiserum may contain high concentrations of antibodies that bind to the antigen but do not induce agglutination; these antibodies, called incomplete antibodies, are often of the IgG class. At high concentrations of IgG, incomplete antibodies may occupy most of the antigenic sites, thus blocking access by IgM, which is a good agglutinin. This effect is not seen with agglutinating monoclonal antibodies. The lack of agglutinating activity of an incomplete antibody may be due to restricted flexibility in the hinge region, making it difficult for the antibody to assume the required angle for optimal cross-linking of epitopes on two or more particulate antigens. Alternatively, the density of epitope distribution or the location of some epitopes in deep pockets of a particulate antigen may make it difficult for the antibodies specific for these epitopes to agglutinate certain particulate antigens.When feasible, the solution to both of these problems is to try different antibodies that may react with other epitopes of the antigen that do not present these limitations.

Hemagglutination Is Used in Blood Typing : Agglutination reactions (Figure 6-7) are routinely performed to type red blood cells (RBCs). In typing for the ABO antigens, RBCs are mixed on a slide with antisera to the A or B blood-group antigens. If the antigen is present on the cells, they agglutinate, forming a visible clump on the slide. Determination of which antigens are present on donor and recipient RBCs is the basis for matching blood types for transfusions.

FIGURE 6-7 Demonstration of hemagglutination using antibodies against sheep red blood cells (SRBCs). The control tube (10) contains only SRBCs, which settle into a solid "button." The experimental tubes 1–9 contain a constant number of SRBCs plus serial two-fold dilutions of anti-SRBC serum. The spread pattern in the experimental series indicates positive hemagglutination through tube 3.

Bacterial Agglutination Is Used To Diagnose Infection : A bacterial infection often elicits the production of serum antibodies specific for surface antigens on the bacterial cells. The presence of such antibodies can be detected by bacterial agglutination reactions. Serum from a patient thought to be infected with a given bacterium is serially diluted in an array of tubes to which the bacteria is added. The last tube showing visible agglutination will reflect the serum antibody titer of the patient. The agglutinin titer is defined as the reciprocal of the greatest serum dilution that elicits a positive agglutination reaction. For example, if serial twofold dilutions of serum are prepared and if the dilution of 1/640 shows agglutination but the dilution of 1/1280 does not, then the agglutination titer of the patient's serum is 640. In some cases serum can be diluted up to 1/50,000 and still show agglutination of bacteria.

The agglutinin titer of an antiserum can be used to diagnose a bacterial infection. Patients with typhoid fever, for example, show a significant rise in the agglutination titer to Salmonella typhi. Agglutination reactions also provide a way to type bacteria. For instance, different species of the bacterium Salmonella can be distinguished by agglutination reactions with a panel of typing antisera.

Passive Agglutination Is Useful with Soluble Antigens: The sensitivity and simplicity of agglutination reactions can be extended to soluble antigens by the technique of passive hemagglutination. In this technique, antigen-coated red blood cells are prepared by mixing a soluble antigen with red blood cells that have been treated with tannic acid or chromium chloride, both of which promote adsorption of the antigen to the surface of the cells. Serum containing antibody is serially diluted into microtiter plate wells, and the antigen-coated red blood cells are then added to each well; agglutination is assessed by the size of the characteristic spread pattern of agglutinated red blood cells on the bottom of the well, like the pattern seen in agglutination reactions (see Figure 6-7).

Over the past several years, there has been a shift away from red blood cells to synthetic particles, such as latex beads, as matrices for agglutination reactions. Once the antigen has been coupled to the latex beads, the preparation can either be used immediately or stored for later use. The use of synthetic beads offers the advantages of consistency, uniformity, and stability. Furthermore, agglutination reactions employing synthetic beads can be read rapidly, often within 3 to 5 minutes of mixing the beads with the test sample. Whether based on red blood cells or the more convenient and versatile synthetic beads, agglutination reactions are simple to perform, do not require expensive equipment, and can detect small amounts of antibody (concentrations as low as nanograms per milliliter).

In Agglutination Inhibition, Absence of Agglutination Is Diagnostic of Antigen: A modification of the agglutination reaction, called agglutination inhibition, provides a highly sensitive assay for small quantities of an antigen. For example, one of the early types of home pregnancy test kits included latex particles coated with human chorionic gonadotropin (HCG) and antibody to HCG (Figure 6-8). The addition of urine from a pregnant woman, which contained HCG, inhibited agglutination of the latex particles when the anti-HCG antibody was added; thus the absence of agglutination indicated pregnancy.

Agglutination inhibition assays can also be used to determine whether an individual is using certain types of illegal drugs, such as cocaine or heroin. A urine or blood sample is first incubated with antibody specific for the suspected drug. Then red blood cells (or other particles) coated with the drug are added. If the red blood cells are not agglutinated by the antibody, it indicates the sample contained an antigen recognized by the antibody, suggesting that the individual was using the illicit drug. One problem with these tests is that some legal drugs have chemical structures similar to those of illicit drugs, and these legal drugs may cross-react with the antibody, giving a false-positive reaction. For this reason a positive reaction must be confirmed by a nonimmunologic method.

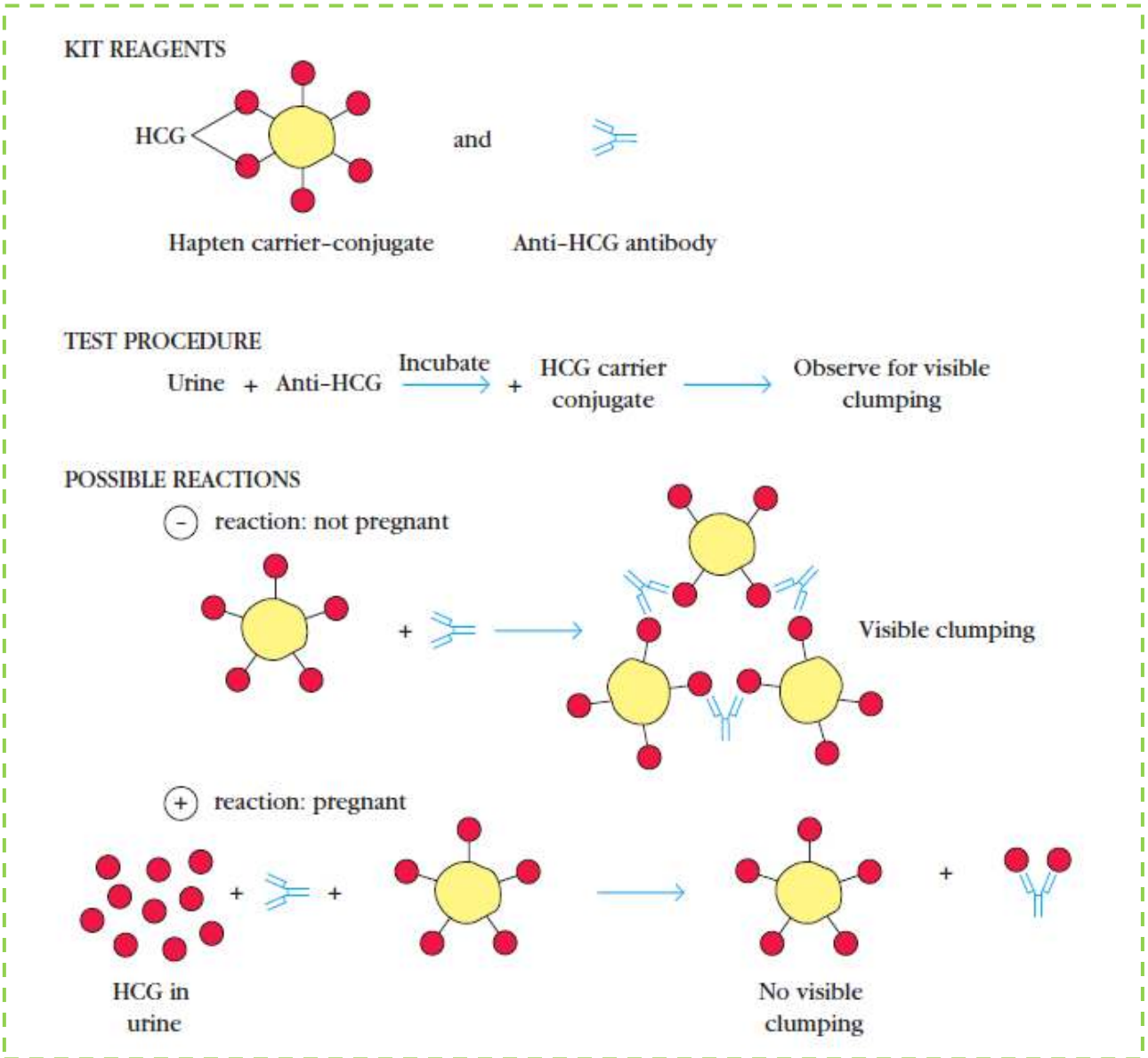


FIGURE 6-8 The original home pregnancy test kit employed hapten inhibition to determine the presence or absence of human chorionic gonadotropin (HCG). The original test kits used the presence or absence of visible clumping to determine whether HCG was present. If a woman was not pregnant, her urine would not contain HCG; in this case, the anti-HCG antibodies and HCG-carrier conjugate in the kit would react, producing visible clumping. If a woman was pregnant, the HCG in her urine would bind to the anti-HCG antibodies, thus inhibiting the subsequent binding of the antibody to the HCGcarrier conjugate. Because of this inhibition, no visible clumping occurred if a woman was pregnant.

Agglutination inhibition assays are widely used in clinical laboratories to determine whether an individual has been exposed to certain types of viruses that cause agglutination of red blood cells. If an individual's serum contains specific antiviral antibodies, then the antibodies will bind to the virus and interfere with hemagglutination by the virus. This technique is commonly used in premarital testing to determine the immune status of women with respect to rubella virus. The reciprocal of the last serum dilution to show inhibition of rubella hemagglutination is the titer of the serum. A titer greater than 10 (1:10 dilution) indicates that a woman is immune to rubella, whereas a titer of less than 10 is indicative of a lack of immunity and the need for immunization with the rubella vaccine.

Radioimmunoassay

One of the most sensitive techniques for detecting antigen or antibody is radioimmunoassay (RIA). The technique was first developed in 1960 by two endocrinologists, S. A. Berson and Rosalyn Yalow, to determine levels of insulin–anti-insulin complexes in diabetics. Although their technique encountered some skepticism, it soon proved its value for measuring hormones, serum proteins, drugs, and vitamins at concentrations of 0.001 micrograms per milliliter or less. In 1977, some years after Berson's death, the significance of the technique was acknowledged by the award of a Nobel Prize to Yalow.

The principle of RIA involves competitive binding of radiolabeled antigen and unlabeled antigen to a high-affinity antibody. The labeled antigen is mixed with antibody at a concentration that saturates the antigen-binding sites of the antibody. Then test samples of unlabeled antigen of unknown concentration are added in progressively larger amounts. The antibody does not distinguish labeled from unlabeled antigen, so the two kinds of antigen compete for available binding sites on the antibody. As the concentration of unlabeled antigen increases, more labeled antigen will be displaced from the binding sites. The decrease in the amount of radiolabeled antigen bound to specific antibody in the presence of the test sample is measured in order to determine the amount of antigen present in the test sample. The antigen is generally labeled with a gamma-emitting isotope such as ^{125}I, but beta-emitting isotopes such as tritium (^{3}H) are also routinely used as labels. The radiolabeled antigen is part of the assay mixture; the test sample may be a complex mixture, such as serum or other body fluids, that contains the unlabeled antigen. The first step in setting up an RIA is to determine the amount of antibody needed to bind 50%–70% of a fixed quantity of radioactive antigen (Ag*) in the assay mixture. This ratio of antibody to Ag* is chosen to ensure that the number of epitopes presented by the labeled antigen always exceeds the total number of antibody binding sites. Consequently, unlabeled antigen added to the sample mixture will compete with radiolabeled antigen for the limited supply of antibody. Even a small amount of unlabeled antigen added to the assay mixture of labeled antigen and antibody will cause a decrease in the amount of radioactive antigen bound, and this decrease will be proportional to the amount of unlabeled antigen added. To determine the amount of labeled antigen bound, the Ag-Ab complex is precipitated to separate it from free antigen (antigen not bound to Ab), and the radioactivity in the precipitate is measured.

A standard curve can be generated using unlabeled antigen samples of known concentration (in place of the test sample), and from this plot the amount of antigen in the test mixture may be precisely determined.

Various solid-phase RIAs have been developed that make it easier to separate the Ag-Ab complex from the unbound antigen. In some cases, the antibody is covalently crosslinked to Sepharose beads. The amount of radiolabeled antigen bound to the beads can be measured after the beads have been centrifuged and washed.Alternatively, the antibody can be immobilized on polystyrene or polyvinylchloride wells and the amount of free labeled antigen in the supernatant can be determined in a radiation counter. In another approach, the antibody is immobilized on the walls of microtiter wells and the amount of bound antigen determined. Because the procedure requires only small amounts of sample and can be conducted in small 96-well microtiter plates (slightly larger than a 3 ——————————— 5 card), this procedure is well suited for determining the concentration of a particular antigen in large numbers of samples. For example, a microtiter RIA has been widely used to screen for the presence of the hepatitis B virus (Figure 6-9). RIA screening of donor blood has sharply reduced the incidence of hepatitis B infections in recipients of blood transfusions.

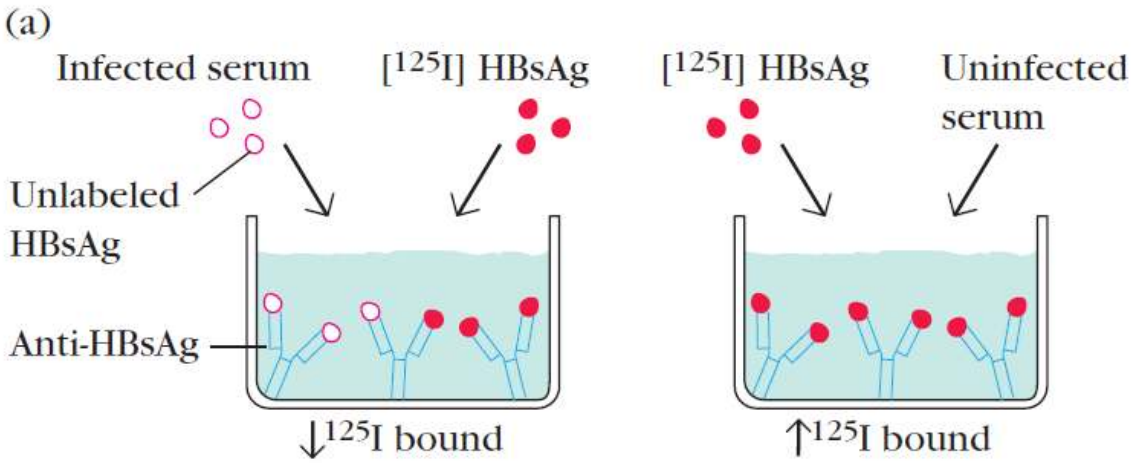


FIGURE 6-9 A solid-phase radioimmunoassay (RIA) to detect hepatitis B virus in blood samples. (a) Microtiter wells are coated with a constant amount of antibody specific for HBsAg, the surface antigen on hepatitis B virions. A serum sample and [125I]HBsAg are then added. After incubation, the supernatant is removed and the radioactivity of the antigen-antibody complexes is measured. If the sample is infected, the amount of label bound will be less than in controls with uninfected serum.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay, commonly known as ELISA (or EIA), is similar in principle to RIA but depends on an enzyme rather than a radioactive label. An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. Such a substrate is called a chromogenic substrate. A number of enzymes have been employed for ELISA, including alkaline phosphatase, horseradish peroxidase, and galactosidase. These assays approach the sensitivity of RIAs and have the advantage of being safer and less costly.

There Are Numerous Variants of ELISA: A number of variations of ELISA have been developed, allowing qualitative detection or quantitative measurement of either antigen or antibody. Each type of ELISA can be used qualitatively to detect the presence of antibody or antigen. Alternatively, a standard curve based on known concentrations of antibody or antigen is prepared, from which the unknown concentration of a sample can be determined.

INDIRECT ELISA: Antibody can be detected or quantitatively determined with an indirect ELISA (Figure 6-10a). Serum or some other sample containing primary antibody (Ab1) is added to an antigen- coated microtiter well and allowed to react with the antigen attached to the well. After any free Ab1 is washed away, the presence of antibody bound to the antigen is detected by adding an enzyme-conjugated secondary anti-isotype antibody (Ab2), which binds to the primary antibody. Any free Ab2 then is washed away, and a substrate for the enzymes added. The amount of colored reaction product that forms is measured by specialized spectrophotometric plate readers, which can measure the absorbance of all of the wells of a 96-well plate in seconds.

Indirect ELISA is the method of choice to detect the presence of serum antibodies against human immunodeficiency virus (HIV), the causative agent of AIDS. In this assay, recombinant envelope and core proteins of HIV are adsorbed as solid-phase antigens to microtiter wells. Individuals infected with HIV will produce serum antibodies to epitopes on these viral proteins. Generally, serum antibodies to HIV can be detected by indirect ELISA within 6 weeks of infection.

SANDWICH ELISA: Antigen can be detected or measured by a sandwich ELISA (Figure 6-10b). In this technique, the antibody (rather than the antigen) is immobilized on a microtiter well. A sample containing antigen is added and allowed to react with the immobilized antibody. After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. After any free second antibody is removed by washing, substrate is added, and the colored reaction product is measured.

COMPETITIVE ELISA: Another variation for measuring amounts of antigen is competitive ELISA (Figure 6-10c). In this technique, antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to an antigencoated microtiter well. The more antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well. Addition of

an enzyme-conjugated secondary antibody (Ab2) specific for the isotype of the primary antibody can be used to determine the amount of primary antibody bound to the well as in an indirect ELISA. In the competitive assay, however, the higher the concentration of antigen in the original sample, the lower the absorbance.

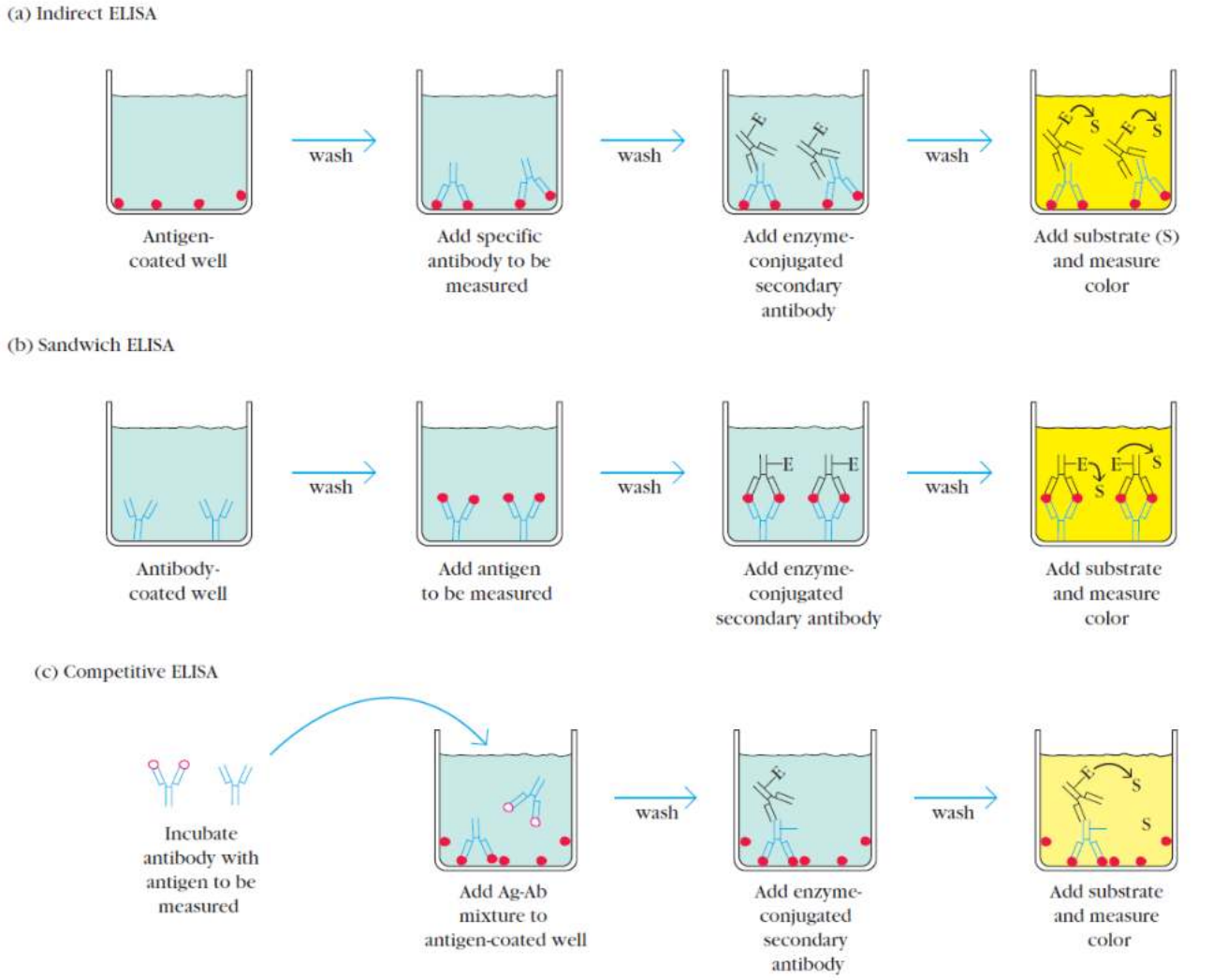


FIGURE 6-10 Variations in the enzyme-linked immunosorbent assay (ELISA) technique allow determination of antibody or antigen. Each assay can be used qualitatively, or quantitatively by comparison with standard curves prepared with known concentrations of antibody or antigen. Antibody can be determined with an indirect ELISA (a), whereas antigen can be determined with a sandwich ELISA (b) or competitive ELISA (c). In the competitive ELISA, which is an inhibition-type assay, the concentration of antigen is inversely proportional to the color produced.

https://www.easybiologyclass.com/enzyme-cell-immobilization-techniques/

Immobilization of Enzymes and Cells: Methods, Effects and Applications

Traditionally, enzymes in free solutions (i.e. in soluble or free form) react with substrates to result in products. Such use of enzymes is wasteful, particularly for industrial purposes, since enzymes are not stable, and they cannot be recovered for reuse.

Immobilization of enzymes (or cells) refers to the technique of confining/anchoring the enzymes (or cells) in or on an inert support for their stability and functional reuse. By employing this technique, enzymes are made more efficient and cost-effective for their industrial use. Some workers regard immobilization as a goose with a golden egg in enzyme technology. Immobilized enzymes retain their structural conformation necessary for catalysis.

There are several advantages of immobilized enzymes:

a. Stable and more efficient in function.

b. Can be reused again and again.

c. Products are enzyme-free.

d. Ideal for multi-enzyme reaction systems.

e. Control of enzyme function is easy.

f. Suitable for industrial and medical use.

g. Minimize effluent disposal problems.

There are however, certain disadvantages also associated with immobilization.

a. The possibility of loss of biological activity of an enzyme during immobilization or while it is in use.

b. Immobilization is an expensive affair often requiring sophisticated equipment.

Immobilized enzymes are generally preferred over immobilized cells due to specificity to yield the products in pure form. However, there are several advantages of using immobilized multi-enzyme systems such as organelles and whole cells over immobilized enzymes. The

immobilized cells possess the natural environment with cofactor availability (and also its regeneration capability) and are particularly suitable for multiple enzymatic reactions.

Methods of Immobilization:

The commonly employed techniques for immobilization of enzymes are—adsorption, entrapment, covalent binding and cross-linking.

Adsorption:

Adsorption involves the physical binding of enzymes (or cells) on the surface of an inert support. The support materials may be inorganic (e.g. alumina, silica gel, calcium phosphate gel, glass) or organic (starch, carboxymethyl cellulose, DEAE-cellulose, DEAE-sephadex).

Adsorption of enzyme molecules (on the inert support) involves weak forces such as van der Waals forces and hydrogen bonds (Fig. 21.3). Therefore, the adsorbed enzymes can be easily removed by minor changes in pH, ionic strength or temperature. This is a disadvantage for industrial use of enzymes.

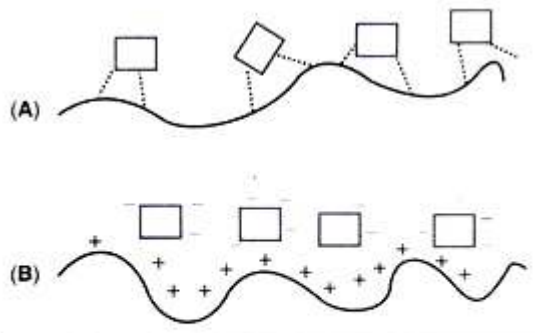


Fig. 21.3 : *Immobilization of enzymes by adsorption (**A**) By van der Waals forces (**B**) By hydrogen bonding (**Note :** Cloured blocks represent enzymes)*

Entrapment:

Enzymes can be immobilized by physical entrapment inside a polymer or a gel matrix. The size of the matrix pores is such that the enzyme is retained while the substrate and product molecules pass through. In this technique, commonly referred to as lattice entrapment, the enzyme (or cell) is not subjected to strong binding forces and structural distortions.

Some deactivation may however, occur during immobilization process due to changes in pH or temperature or addition of solvents. The matrices used for entrapping of enzymes include polyacrylamide gel, collagen, gelatin, starch, cellulose, silicone and rubber. Enzymes can be entrapped by several ways.

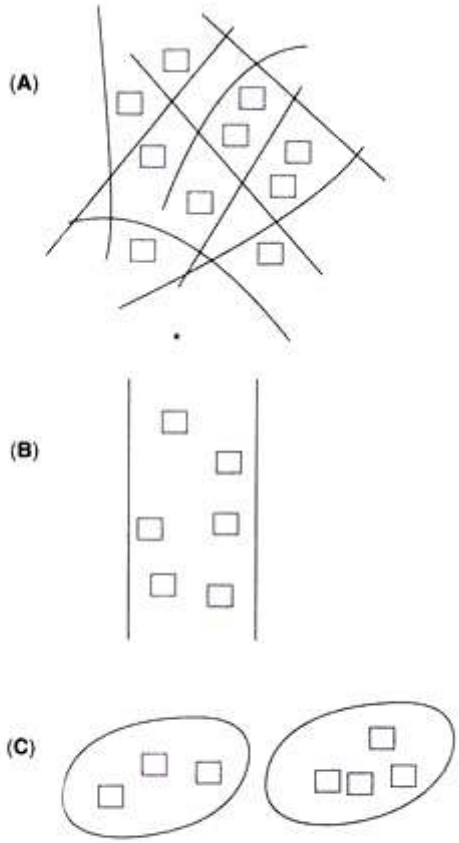


*Fig. 21.4 : Immobilization of enzymes by entrapment (A) Inclusion in gels (B) Inclusion in fibres (C) Inclusion in microcapsules (**Note** : Coloured blocks represent enzymes)*

1. Enzyme inclusion in gels:

This is an entrapment of enzymes inside the gels (Fig. 21.4A).

2. Enzyme inclusion in fibres:

The enzymes are trapped in a fibre format of the matrix (Fig. 21.4B).

Preparation and Properties of an Immobilized Derivative of Penicillinase

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Summary

Penicillinase (β-lactamase, E.C. 3.5.2.6), secreted by *Bacillus cereus* strain 569/H, was covalently attached to [illegible] phenolic resin. The immobilized derivative [illegible] conformational changes induced [illegible] penicillins. [illegible] *Bacillus cereus* [illegible] the conformational flexibility of the enzyme molecule. This [illegible] immobilized penicillinase [illegible] for the study of the role of flexibility in the catalytic [illegible] enzymes.

INTRODUCTION

Immobilized enzymes are widely used in studies investigating the mechanisms of enzyme action and in the field of enzyme technology. The enzyme is usually immobilized by binding the soluble protein molecules to an insoluble matrix via those groups and residues that are not essential in catalysis. Nevertheless, changes in the molecular properties of the enzyme, such as its stability and allosteric sensitivity, have been observed.[1-4] Such changes suggest that the flexibility of the protein conformation is restricted as a result of the binding. Previous studies of the penicillinase secreted by *Bacillus cereus* (β-lactamase, E.C. 3.5.2.6)[5-8] have shown that this enzyme might be especially suitable for the study of the functional consequences of immobilization and of the restriction of flexibility introduced by the process. The enzyme catalyzes the hydrolysis of the β-lactam ring in 6-aminopenicillanic acid and its derivatives. It was shown that the enzyme molecule is highly flexible and undergoes conformational changes upon interaction with the substrates

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0006-3592/79/0021-0897$01.00

[conformational] enzyme.[14] These substrates can be classified as S- or R-type penicillins which differ from one another by the way they induce in the enzyme. Thus, an S-type penicillin induces a conformation that is unfavorable for the reaction. The main role of the conformational response in penicillinase appears to be protective, making it a convenient model for the study of the effect of restricted flexibility on the conformational properties of proteins. In the present work described is the modification of penicillinase by covalent attachment to polyethylene glycol monomethyl carbonate via the 4,6-dichloro-s-triazinyl reagent (cyanuric chloride). The properties of the derivatives obtained from the point of view of immunization and conformational restriction by modification.

MATERIALS AND METHODS

Benzylpenicillin was obtained from Eli Lilly Laboratories. 6-Aminopenicillanic acid was purchased from Aldrich Chemical Co. All other reagents were obtained from Merck Laboratories. Glutaraldehyde (25%) was obtained from Ladd Laboratories. Amberlite IRA-400 (Cl form) was the product of Rohm and Haas Co. All other reagents were of analytical grade.

Penicillinase Preparation

The exopenicillinase of *Bacillus cereus* 569/H was purified according to previously published procedures.[15] One enzyme unit is the amount of enzyme that hydrolyzes 1 μmol of benzylpenicillin in 1 min at 30°C, pH 7.0.[16]

Penicillinase Assay

The various methods used to determine the activity of the soluble and the immobilized enzyme were described previously.[17] A modified procedure of the Chalmers assay[18] was used in this work. The enzyme was added to a solution of final volume 3 ml containing glycine-phosphate buffer (0.1 *M*, pH 7.0), albumin (0.25%), and substrate. The pH was kept constant by titration with NaOH (0.05 *N*) using an Autoburette.

Enzyme Immobilization

The enzyme was bound to aminoethyl cellulose by the method of Glossmann and Neville[19] with some modifications. In the first stage,

900 mg aminoethyl cellulose were washed twice with 5 ml 5% NaOH and twice with 10 ml 10% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0. After 2 hr incubation with stirring at room temperature, excess glutaraldehyde was removed by repeat washes with 0.1 M phosphate buffer, pH 7.0.

In the second stage, 1.50 mg enzyme were added to 250 mg activated cellulose in 10 ml 0.1 M phosphate buffer, pH 7.0. The suspension was stirred for 2 hr at room temperature and then washed several times with a solution of 1 M NaCl in 0.1 M phosphate buffer, pH 8.0, in order to wash out any absorbed enzyme. The bound enzyme was kept at 4°C in 5 ml 0.1 M phosphate buffer, pH 7.0. No activity was detected in the supernatant even after three hours of storage.

Determination of Michaelis Constant for 6-Aminopenicillanic Acid

A modified spectrophotometric method was used for the determination of K_m for the immobilized enzyme and free enzyme in the case of 6-APA. The reaction mixture was a volume 20 ml containing immobilized enzyme and 6-APA. pH was maintained at 7.0 with stirring. Samples (2 ml) were withdrawn every 2 min, cooled immediately, and filtered in vacuum at 4°C to remove the enzyme. The hydrolyzed substrate in each sample was determined by its absorbance at 240 nm. The K_m was calculated according to Lineweaver and Burk.[9]

RESULTS

Immobilization of the Enzyme to Cellulose

In the coupling procedure used (see "Materials and Methods"), one of the groups of the bifunctional reagent is bound to the insoluble carrier, while the other group remains free to bind to NH_2 residues of the protein. The optimal ratio between the enzyme and the carrier was determined by comparing the activity obtained with a constant amount of enzyme and various amounts of the carrier (see above). Maximal binding was achieved when 200–300 mg of the carrier were used to bind 125 mg enzyme protein (Fig. 1). The covalent nature of the binding was demonstrated by several washings of the bound enzyme with 1 M NaCl in 0.1 M phosphate buffer, pH 8.0 (see "Materials and Methods"). No substrate solution (benzylpenicillin, 50 mg/ml) No enzyme activity was detected in the supernatant after the washings, while all the activity was washed out

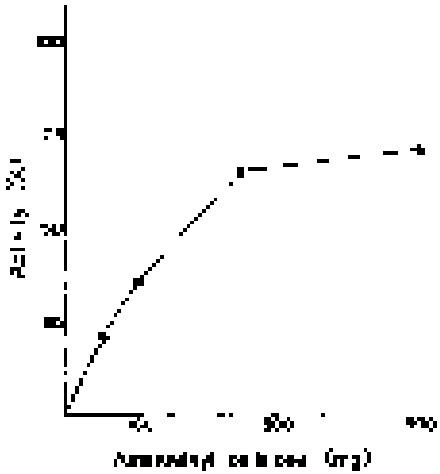

FIG. [illegible]—Inactivation of immobilized penicillinase at 60° and subsequent reactivation by different amounts of glycine ethyl ester at pH 7.0. Immobilized penicillinase was prepared by coupling penicillinase (1,000 units/mg) to aminoethyl cellulose (1 mg of protein/g of wet cellulose) as described by Weetall.[illegible] Activity of the immobilized enzyme was determined by the procedure of Nelson and Griffin.[illegible] One unit of enzyme activity

when the enzyme was adsorbed on aminoethyl cellulose that was not first coated with glutaraldehyde (data not shown).

Modification of Enzyme Activity

The stabilization of proteins on hydrophilic carriers was shown in many cases to increase their stability toward heat denaturation.[illegible] In such cases, the covalent attachment to the insoluble carrier prevents the thermal destruction of the tertiary structure of the protein. Increased thermostability of immobilized penicillinase is demonstrated in Figure 1. A more specific test of immobilization by attachment is summarized in Figure 5. It is based on previous observations[illegible] that mechanillin, and all other R-type penicillins, induce in penicillinase the inactivating of an essential tyrosine residue. Iodination of the exposed residue causes loss of activity. As shown in Figure 5, the immobilized derivative is more resistant toward mechanillin-induced inactivation than the native enzyme. This is not the result of lower affinity of the enzyme derivative for mechanillin; as shown in Figure 5, its effect on iodination does not increase substantially at higher ligand concentrations.

Kinetics of Hydrolysis of R-type Substrates

The kinetics of hydrolysis of R-type penicillins (cf. Table I, which are 6-phenoxy-substituted penicillins, could be affected by the conformational changes induced in the enzyme by these substrates.[15] We expected that a constraint imposed on the flexibility of the enzyme will prevent such changes and therefore suppress the biphasic kinetics. Hydrolysis of R-type substrates such as penicillin V and oxacillin by native and immobilized enzymes was determined by the spectrophotometric and the alkalimetric assays, respectively (Table I). The activity of the immobilized enzyme cannot be followed by the spectrophotometric assay because of the high turbidity of the preparation. On the other hand, the alkalimetric assay is unsuitable for R-type penicillins and the native enzyme since these substrates inhibit the enzyme to a high degree.[4] The two assays are, however, equally applicable to the soluble (native) enzyme with benzylpenicillin as R-type penicillin as the substrate, and are equally reliable.

The results in Table I show that immobilization suppresses the

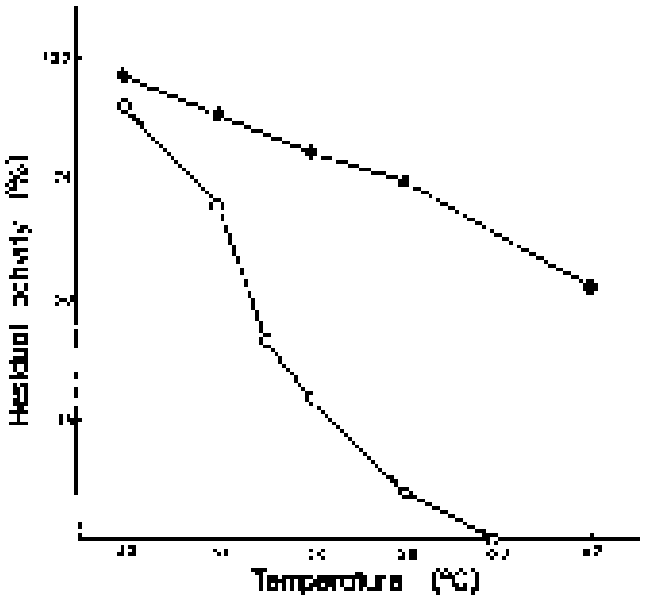

FIG. 1. Thermal deactivation of native and immobilized R-type penicillinase. Samples (0.1 ml) of 0.5% penicillinase solution (native enzyme) or of immobilized enzyme suspended in 0.2% gelatin in phosphate buffer (0.05 M, pH 7.5) were incubated for 5 min at the indicated temperatures and then cooled for 1 min at 0°C. Residual activity was determined by the iodometric method and recalculated as percentage of the initial activity.

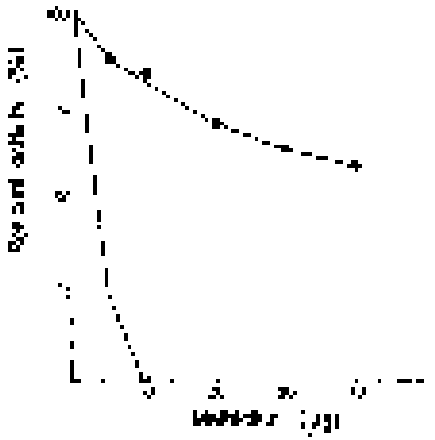

FIG. 1. Stability of penicillinase immobilized on cyanide-activated Sepharose 4B at 37°C. Immobilized enzyme was incubated in 0.05 M phosphate buffer, pH 7.0, containing 0.1 M NaCl. Aliquots were removed at various times and assayed for penicillinase activity. Activity is expressed as a percentage of the initial activity. Each point represents the average of duplicate assays. Half-life was calculated to be 5 days (120 hr). Reactivity was lost rapidly when the immobilized enzyme was exposed to the presence of the protein-denaturing agent, 8 M urea.

hydrolyze substrates, undoubtedly because it constrains the conformational flexibility of the enzyme. The effect of "normal" penicillins on K_m values for several substrates is shown in Table II. The values were derived from Lineweaver–Burk double reciprocal plots which showed no deviation from Michaelis–Menten kinetics.

The conformational restraint hypothesis predicts increases in K_m for all substrates except 6-APA, and is predicated on the basis of conformational constraints.[17] However, in an immobilized enzyme, an apparent increase in K_m may also arise from hindered accessibility of the substrate to the active site.

DISCUSSION

Partial loss of catalytic activity and changes in several of the other properties of the enzyme were observed when penicillinase was immobilized by aminoethyl cellulose. The increased heat resistance of the immobilized derivative suggests that the immobilization stabilized the tertiary structure of the protein. Evidence against this conclusion was suggested by Neumann[18] that the conformational changes may occur when penicillinase is exposed to 4 M urea solu-

TABLE I
Relative Rates of Hydrolysis of Native and Immobilized Penicillinase of SPA [illegible]

Substrate	Type	Rate of hydrolysis			
		Native enzyme		Immobilized enzyme	
Benzylpenicillin	S	[illegible]		[illegible]	
Ampicillin	S	157		[illegible]	
Phenoxymethylpenicillin	S	[illegible]		[illegible]	
Methicillin	A	[illegible]	22	[illegible]	[illegible]
Oxacillin	A	[illegible]	21	[illegible]	[illegible]
Cloxacillin	A	[illegible]	6.4	[illegible]	[illegible]

[a] The rates of hydrolysis of various penicillins by the native enzyme were determined by the [illegible] method [illegible]. For the immobilized enzyme the following methods were used: [illegible]

[illegible]

[illegible]

[illegible] The [illegible] values of hydrolysis of penicillins [illegible] were also determined with [illegible] of penicillinase. As expected, the [illegible] in direct proportion to the degree of immobilization of the enzyme.

changes are similar to those that occur upon exposure to elevated temperatures. We therefore tested whether the alteration of the tertiary structure by immobilization also restricts the conformative response to these types of substrates. The following results indicate that the conformative response of the immobilized enzyme is constrained.

(1) Inactivation of the native enzyme caused by incubation at a [illegible] which is prevented in the presence of A-type substrates is largely prevented.

TABLE II
Michaelis Constants (mM) of Native and Immobilized Penicillinase Preparations

Substrate	Native enzyme[a]	Immobilized enzyme[b]
Benzylpenicillin	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
Methicillin	[illegible]	[illegible]

[a] Assayed by the [illegible] method of [illegible].

[b] Assayed manometrically.

[illegible] "Materials and Methods."

[illegible] the [illegible] kinetics characteristic of the hydrolysis of [illegible] substrates is suppressed. Susceptibility to [illegible] and [illegible] [illegible] have been shown to reflect conformational changes [illegible] by [illegible] substrates [illegible] be active site of the enzyme.

The effect of immobilization on the K_m value poses an interesting question. Does the first-order [illegible] simply [illegible] [illegible] [illegible] of diffusion? These two pairs of curves [illegible] the [illegible] K_m values for all penicillins tested are significantly lower than the K_m for the unsubstituted penicillin substrate, 6-APA. This was not expected if the R-acyl substituents provide [illegible] which [illegible] improve the affinity for the enzyme. Evidence has been presented that the observed affinity is also caused by a [illegible] [illegible] formation of the active site; if this change is prevented, then [illegible] for all such substrates are [illegible] and [illegible] [illegible] effect of immobilization on K_m can be similarly attributed to the rapidity [illegible] [illegible] [illegible]. Although [illegible] the high K_m values observed after immobilization reflect a [illegible] alignment of the binding domain which is now conformationally restricted.

Alternative explanations must [illegible] the need for conformational flexibility must also be considered. In the present heterogeneous system, accessibility of the enzyme to the substrates may be [illegible]. Changes in [illegible] between the substrates and the matrix have been ruled out by [illegible] that the initial rates of the reaction at different [illegible] concentrations (K_m or [illegible]) were not affected by altering the ionic strength of the system (data not shown). Diffusion limitations of the active site of the immobilized [illegible] should [illegible] [illegible] although in such cases the K_m for 6-APA should also increase. Also, no emulsion [illegible] were observed when substrates close in size to the penicillins were hydrolyzed by an [illegible] [illegible] prepared in a similar way.

Our data strongly support the hypothesis wherein any enzymatic activity is retained when the conformative response is completely eliminated. All the derivatives that were [illegible] active showed a certain degree of conformational flexibility. Although highly susceptible [illegible] [illegible] of the [illegible] [illegible], it is not unlikely that this residual flexibility is essential for catalytic function.

References

1. [illegible]
2. [illegible]
3. [illegible]

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Antigen and Antibody Reactions

Detection By

Precipitation Methods

Dr.T.V.Rao MD

Beginning of Serology

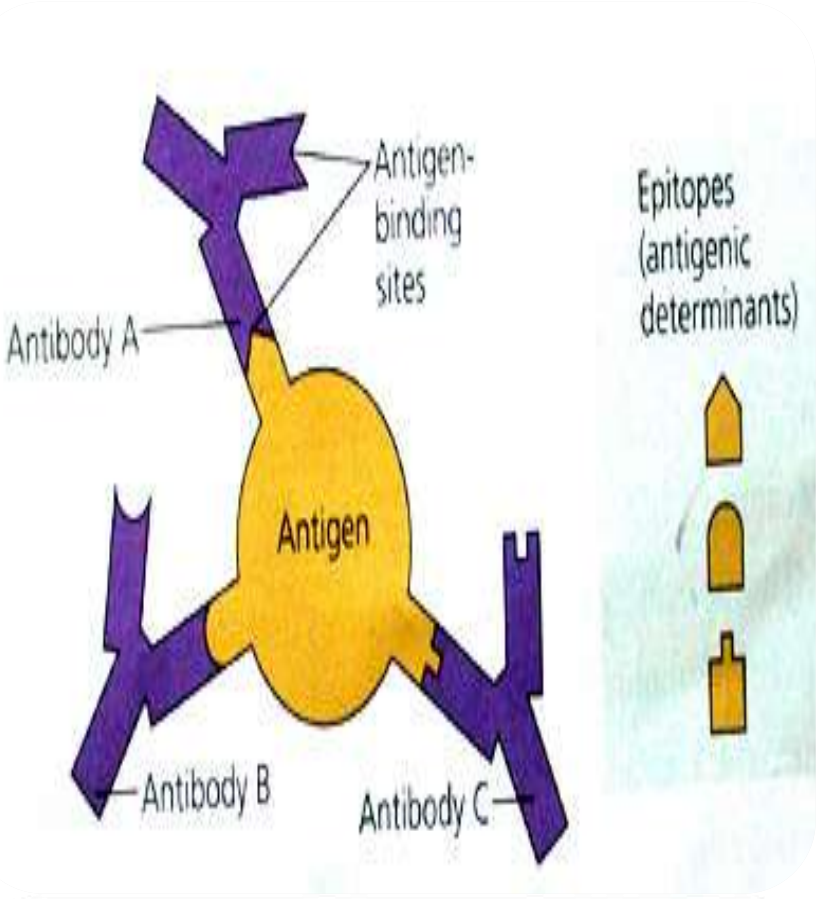
- Serology as a science began in 1901. Austrian American immunologist **Karl Landsteiner** (1868-1943) identified groups of red blood cells as A, B, and O. From that discovery came the recognition that cells of all types, including blood cells, cells of the body, and microorganisms carry proteins and other molecules on their surface that are recognized by cells of the **immune system**.

Characteristics of Antigens

- **Immunogenicity** – property of substance (**immunogens** or **antigens**) to induce a detectable immune response
- **Antigenic specificity** – property of antigen molecule (or its part) to react with the **specific antibody**.
- **Antigenicity** – given by a surface structure of immunogen - **antigenic determinants**. The organism responds only to those that are foreign to him.
- **The number of antigenic determinants** – usually varies with the size and chemical complexity of macromolecule (egg ovalbumin, MW 42 000, has 5 antigenic determinants and thyroglobulin, MW 700 000, has many as 40).

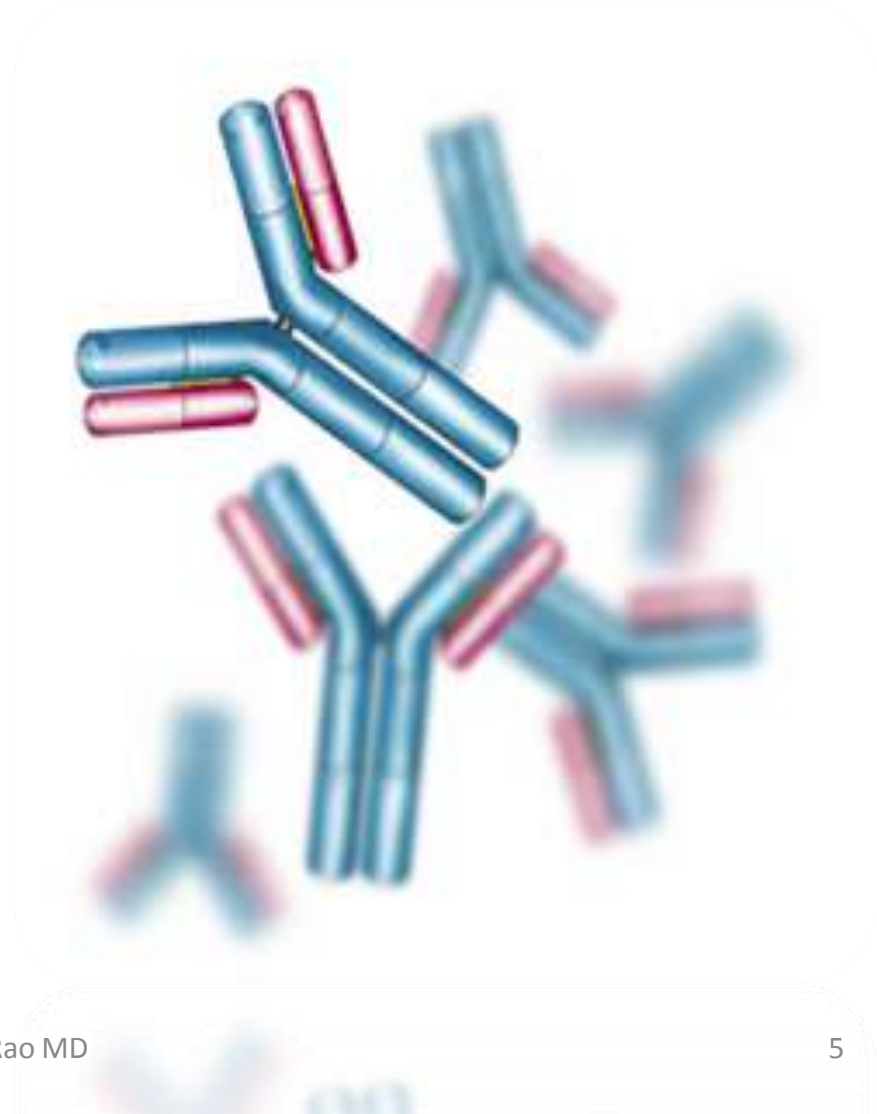
Characteristics of Antigens

- **Chemical nature of antigens:**
  - proteins
  - polysaccharides
  - lipopolysaccharides
  - nucleoproteins
  - glycoproteins
  - steroid hormones
  - bacterial cells, viruses
  - synthetic polypeptides
  - synthetic polymers



Characteristics of Antibodies (Immunoglobulins)

- Proteins with the property of specific combination with antigen (or one antigenic determinant) which elicited their formation.
- Immunoglobulin's account for ~ 20% of the total plasma proteins.

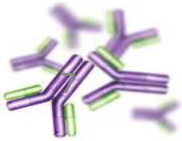


Karl Landsteiner (1868-1943)

- An Austrian physician by training, Landsteiner played an integral part in the identification of blood groups. He demonstrated the catastrophic effect of transfusing with the wrong type of blood,

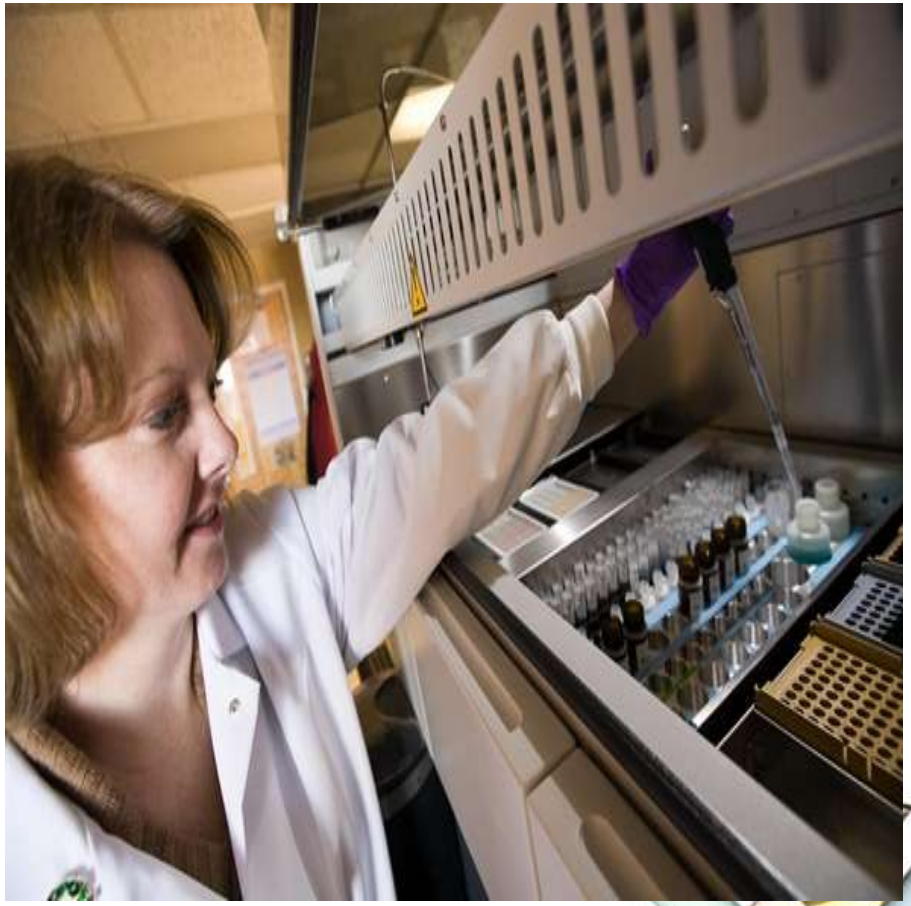
Purpose of Serological Tests

- Serological tests may be performed for diagnostic purposes when an infection is suspected, in rheumatic illnesses, and in many other situations, such as checking an individual's blood type. Serology blood tests help to diagnose patients with certain immune deficiencies associated with the lack of antibodies, such as X-linked agammaglobulinemia.



Serology

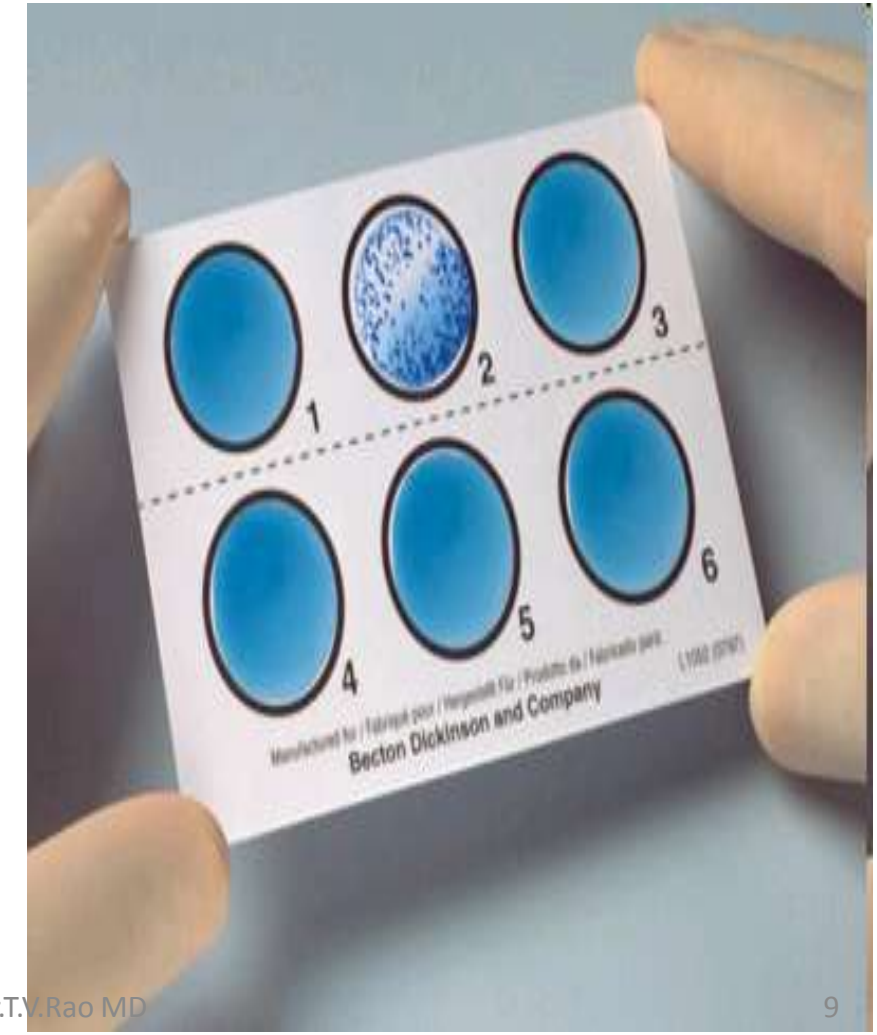
- The branch of laboratory medicine that studies blood serum for evidence of infection and other parameters by evaluating antigen-antibody reactions in **vitro**



Serology

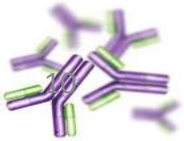
- **Serology** is the scientific study of blood serum. In practice, the term usually refers to the diagnostic identification of antibodies in the serum

We can detect antigens too



Serology Prerogative of Microbiology

- It is rather curious that, although serum for a multitude of constituents in biochemistry and haematological laboratories, the term serology has come to imply almost exclusively the detection of antibodies in serum for ***antibodies in infectious diseases***, and terminology has become prerogative of microbiologists.



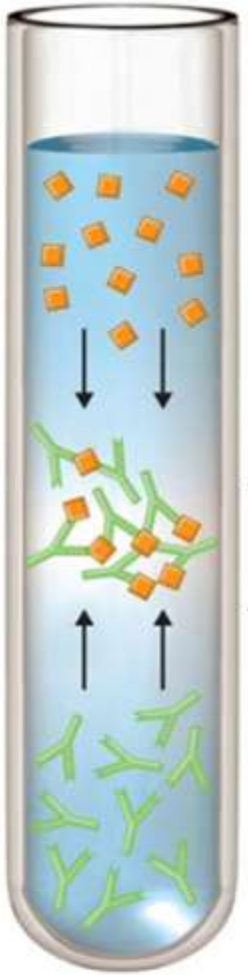
Immunological Tests

- A harmful agent can be detected
  - Toxins, viruses, hard to culture bacteria
  - Takes advantage of the specific nature of antibodies
- Rise in amount of antibody is an indicator
  - An increase in specific antibody is an indicator of exposure, can confirm a diagnosis.
- Antibodies bind, precipitate, and agglutinate.

Precipitation tests

- When sufficient antigen and antibody molecules interact, they precipitate out of solution
  - Too few antigen molecules, little ppt.
  - Too many, agn-aby cross links not made.
- Examples
  - immunodiffusion: antibody and antigen react in agar to make ppt band or ring.
  - Immunoelectrophoresis: complex mixture of antigens separated, then reacted with antibody.

Precipitation Reaction as happens in VITRO



Antigens (soluble)

Zone of equivalence: visible precipitate

Antibodies

(a)

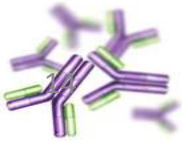
Precipitation band

(b)

Immunology/ Serology?
Precipitation Reactions

- Capillary tube precipitation (Ring Test)
- Ouchterlony Double Diffusion (Immunodiffusion)
- Radialimmunodiffusion (RID)
- Immunoelectrophoresis (IEP)
- Rocket Electroimmunodiffusion (EID)
- Counterimmunoelectrophoresis (CIEP)

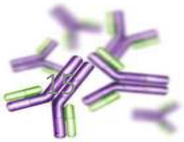
The above tests have moved to Biochemistry



Terms used in evaluating test methodology

Sensitivity

- **Analytical Sensitivity** – ability of a test to detect very small amounts of a **substance**
- **Clinical Sensitivity** – ability of test to give positive result if patient has the disease (no false negative results)

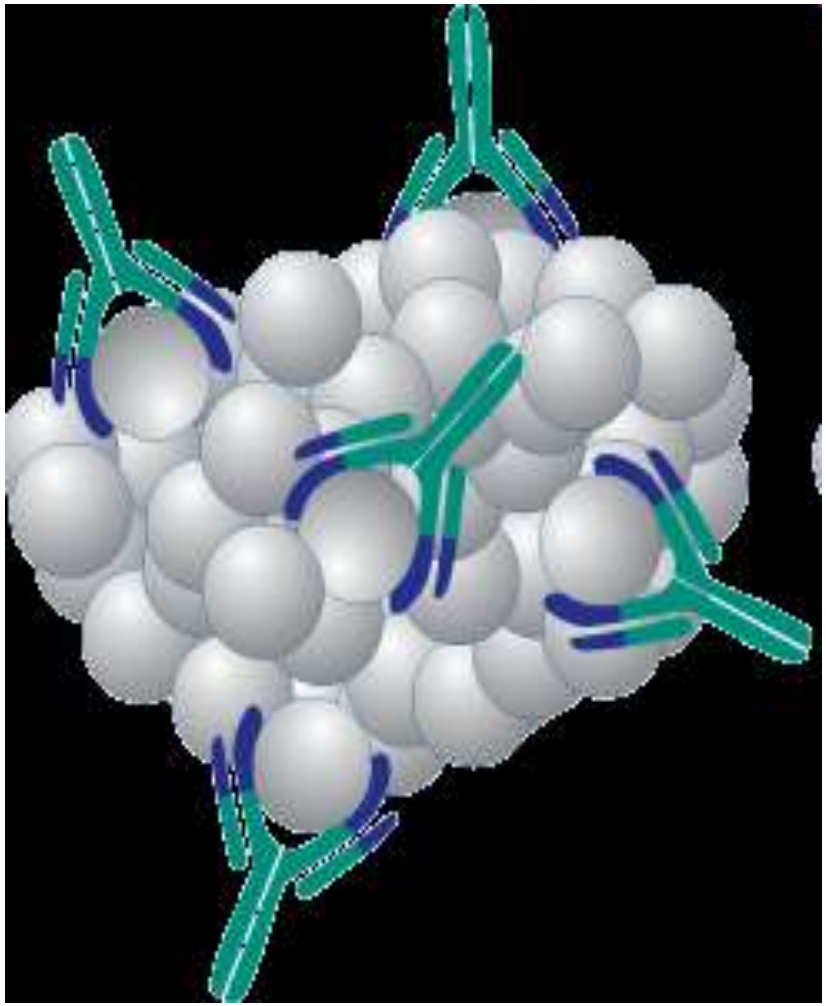


Specificity

- **Analytical Specificity** – ability of test to detect substance without interference from cross-reacting substances
- **Clinical Specificity** – ability of test to give negative result if patient does not have disease (no false positive results)

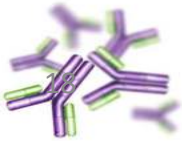
Affinity

- Affinity refers to the strength of binding between a single antigenic determinant and an individual antibody combining site.
- Affinity is the equilibrium constant that describes the antigen-antibody reaction

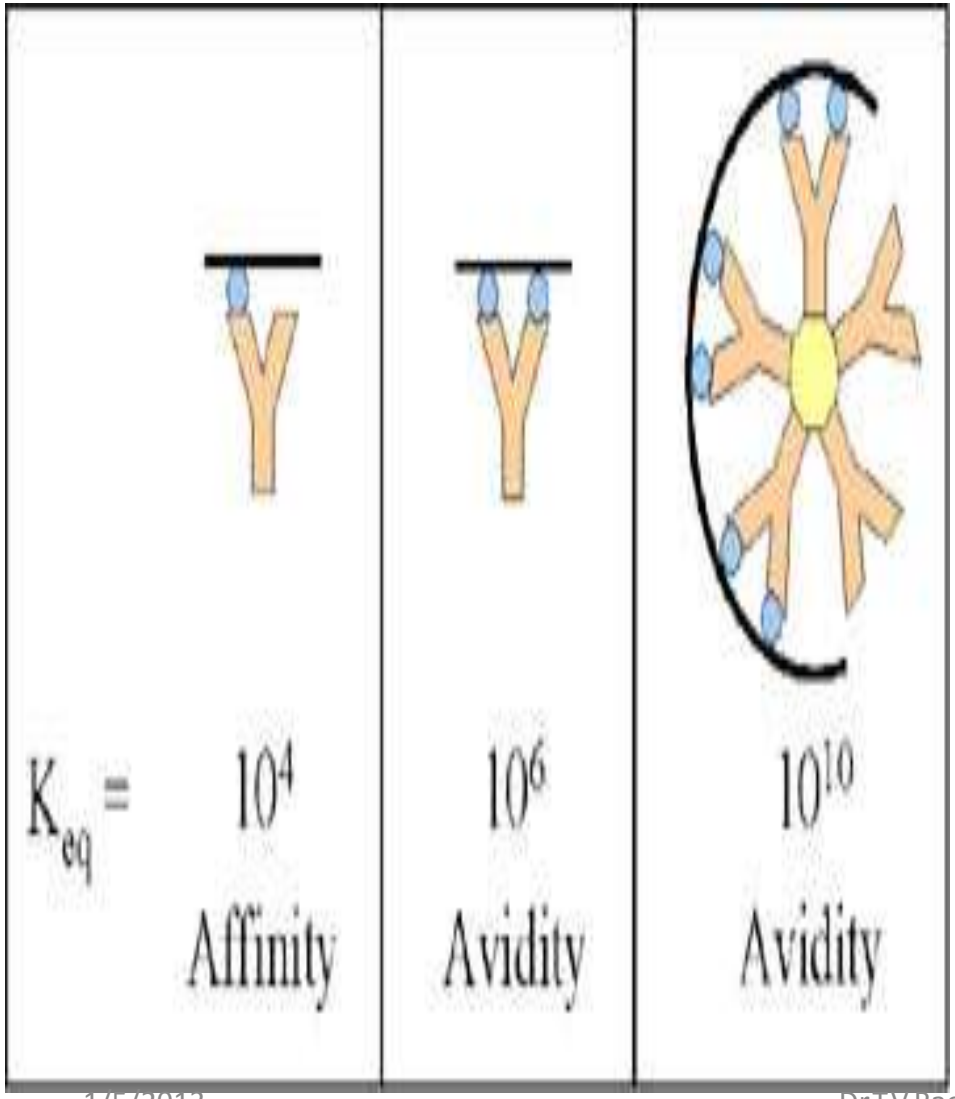


Affinity

- Antibody affinity is the strength of the reaction between a single antigenic determinant and a single combining site on the antibody.
- It is the sum of the attractive and repulsive forces operating between the antigenic determinant and the combining site .

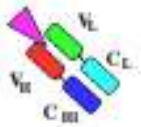
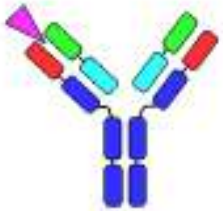
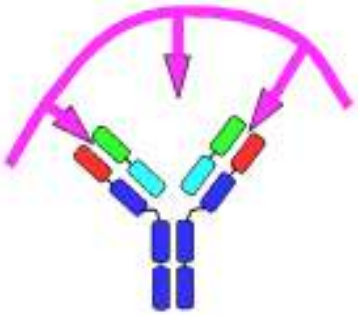
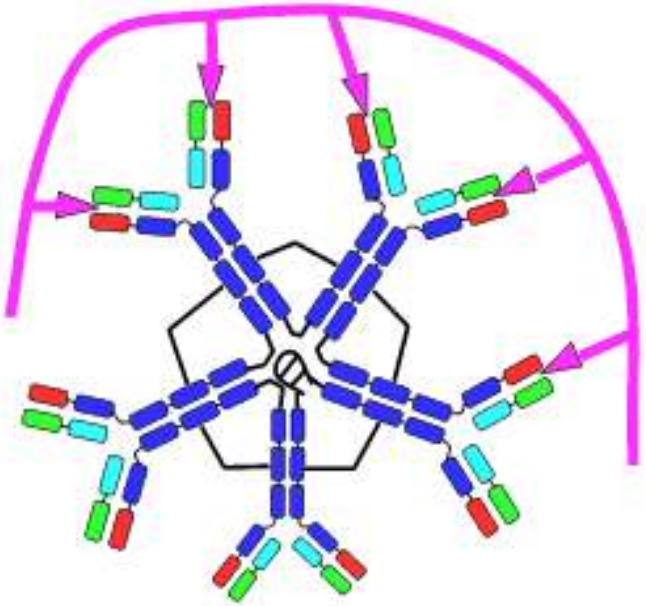


Avidity



- **Avidity is a measure of the overall strength of binding of an antigen with many antigenic determinants and multivalent antibodies**
- Avidity is influenced by both the valence of the antibody and the valence of the antigen.
- **Avidity is more than the sum of the individual affinities.**

Affinity and avidity

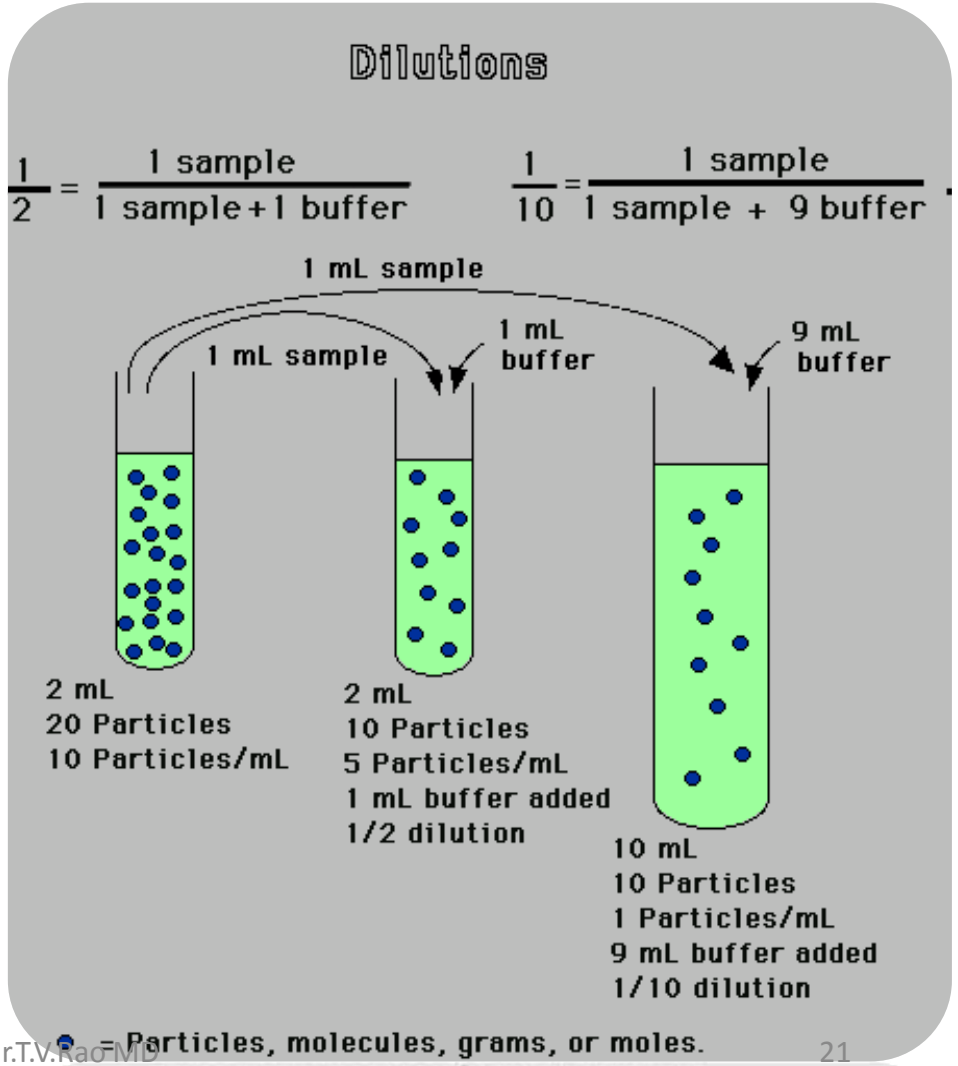
Fab	IgG	IgG	IgM	
				
1	1	2	5	effective valence
1	1	100	10^4 - 10^5	relative equilibrium constant

monovalent affinity

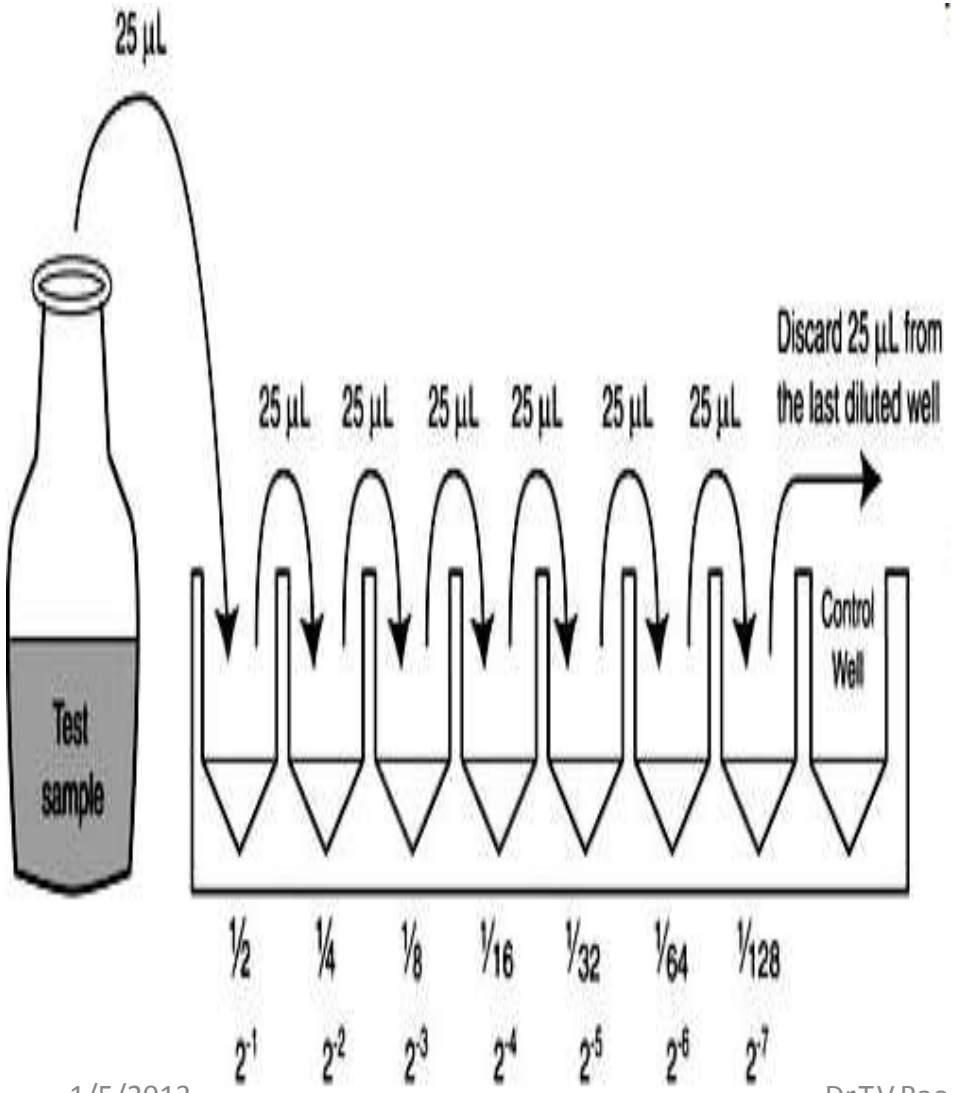
multivalent avidity

Dilution

- Estimating the antibody by determining the greatest degree to which the serum may be diluted without losing the power to given an observable effect in a mixture with specific antigen



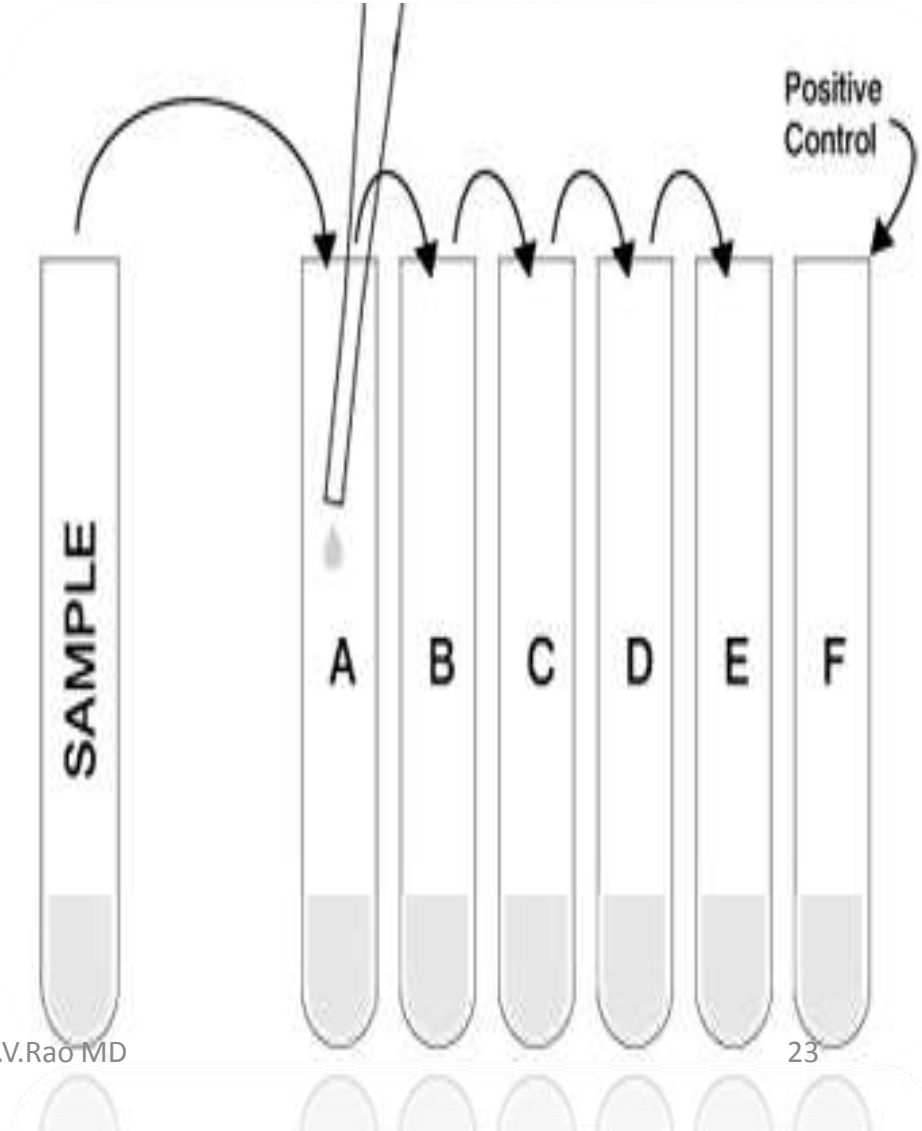
Titer



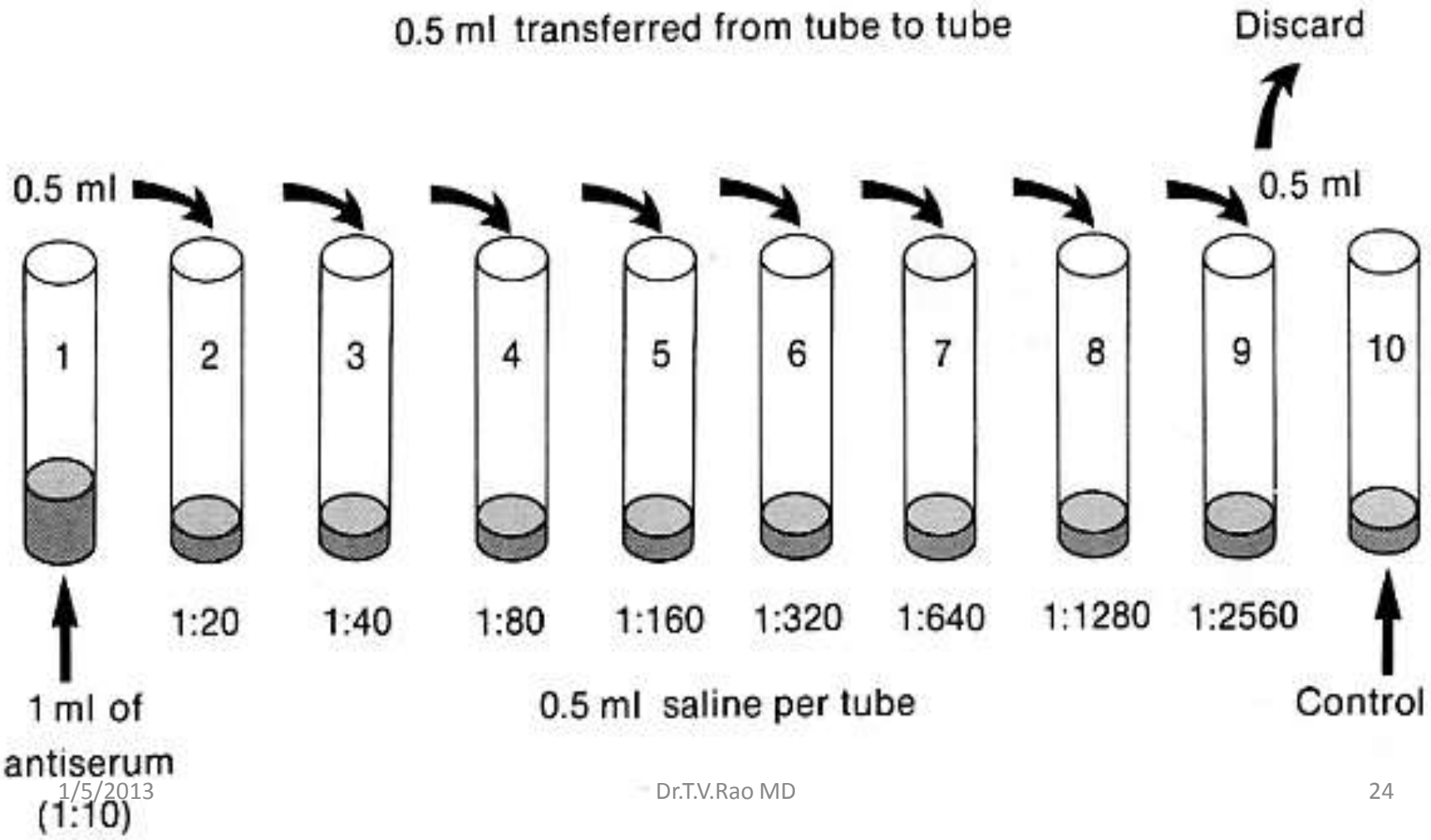
- Different dilutions of serum are tested in mixture with a constant amount of antigen and greatest reacting dilution is taken as the measure or Titer

Expression of Titers

- Expressed in term of the was in which they are made
- Dilution **1 in 8 is a dilution made by mixing one volume of serum with seven volumes of diluents** **(Normal Saline)**
- **Incorrect to express dilution as 1/8**



Common methods in creating dilutions



Characteristics of Antibodies are Variable (Immunoglobulins)

- Variability of antibodies is subject to 5-classes of Ig: G, A, M, D, E
- Heavy chains – γ, α, μ, δ, ε
- Light chains – κ, λ
- Subclasses of immunoglobulins:
  - IgG – γ_1, γ_2, γ_3, γ_4
  - IgA – α_1, α_2
  - IgM - μ_1, μ_2

The forces binding antigen to antibody

- **Electrostatic** : between attraction oppositely charged ionic group – ($-NH_3^-$) of lysine and ($-COO^-$) of aspartate.
- **Hydrogen bonding** – relatively weak and reversible hydrogen bridges between hydrophilic group (-OH, $-NH_2$, COOH).
- **Hydrophobic**– non-polar, hydrophobic side chains of Val, Leu, Ile (hydrophobic groups come close together and exclude water molecules between them. The force of attraction increases.
- **Van der Waals** – forces which depend upon interaction between the external „electron clouds“. Non-specific attractive forces.

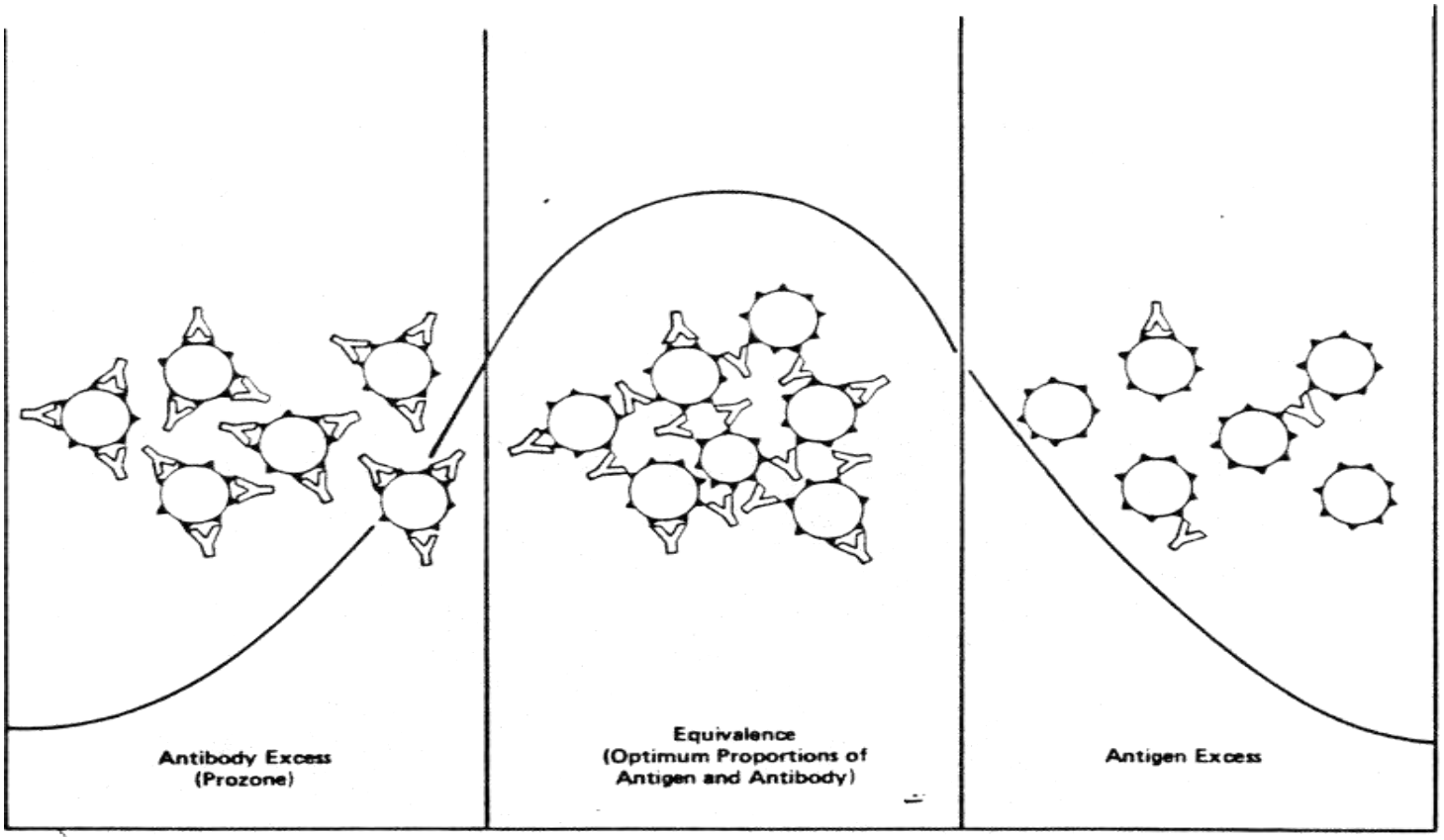
Antibody Affinity

$$Ab + Ag \underset{k_1}{\overset{k_2}{\leftrightarrows}} AbAg$$

$$K = \frac{[AbAg]}{[Ab]\,[Ag]} \quad \frac{k_1}{k_2}$$

Equilibrium constant

The ratio of antigen / antibody

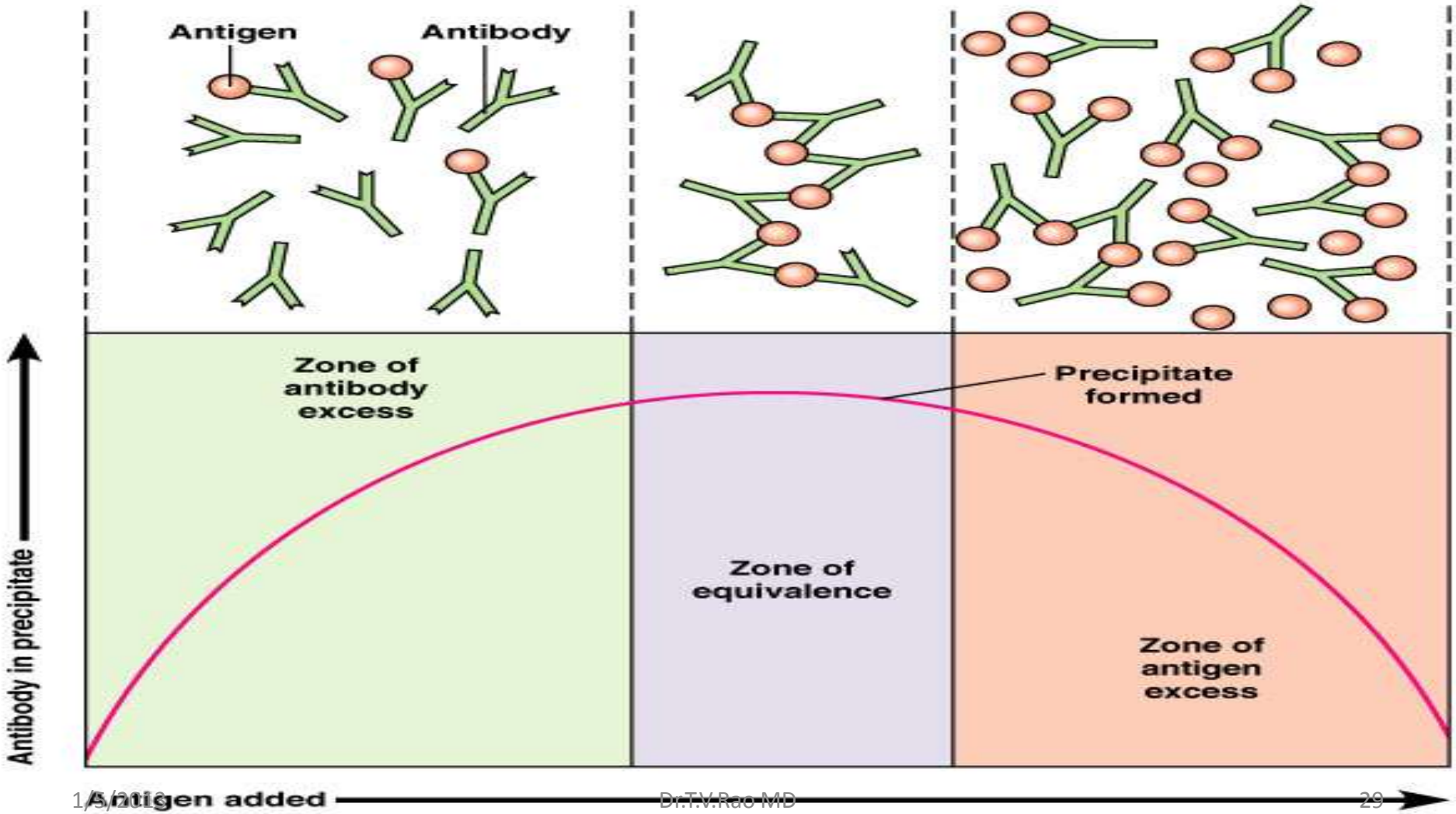


Prozone : Ab excess, precipitate does not form ((soluble immune complexes)

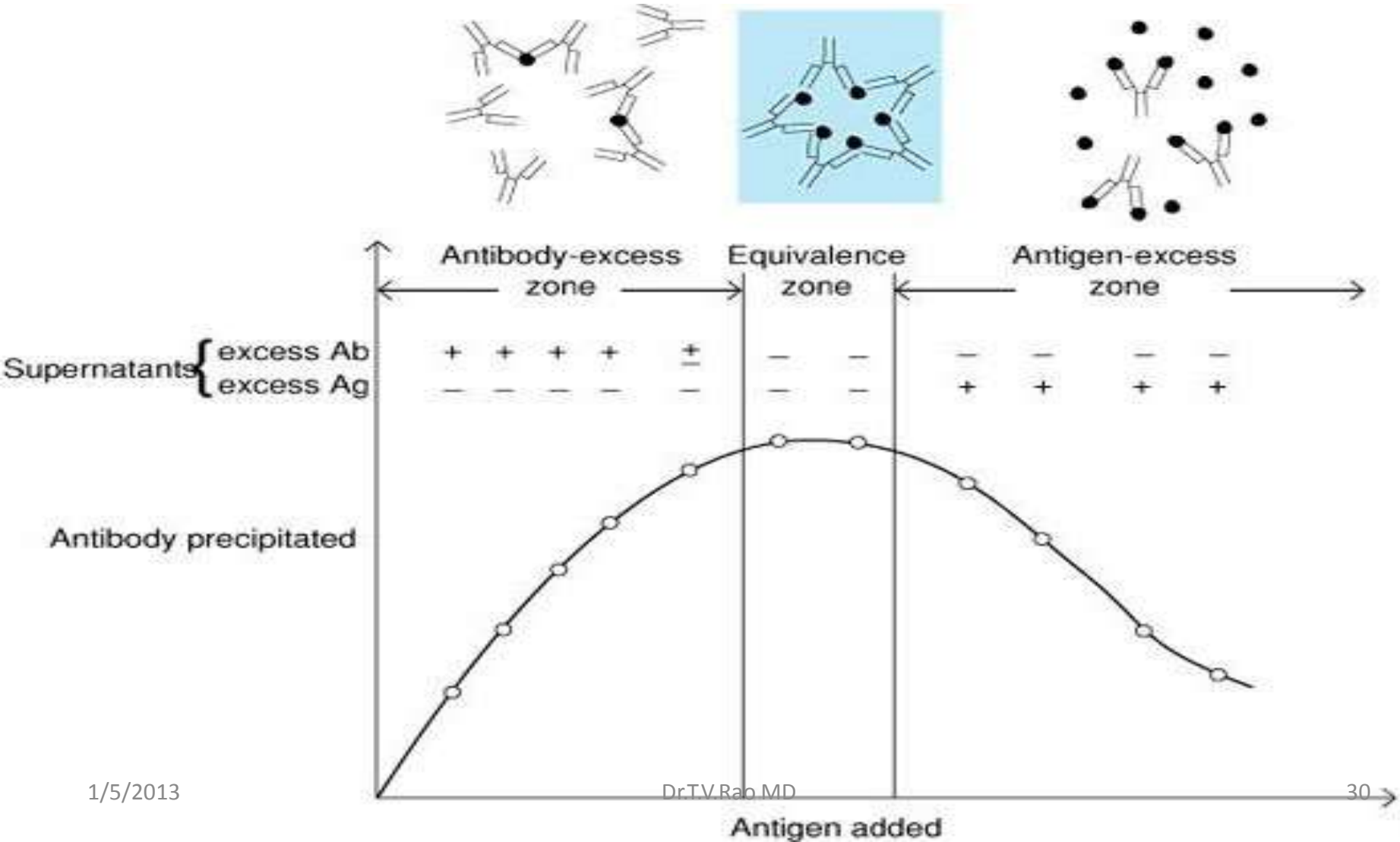
Zone of equivalence- optimal ratio of Ag/Ab – insoluble precipitate

Post-zone – excess of Ag (soluble immune complexes)

Precipitation Curve

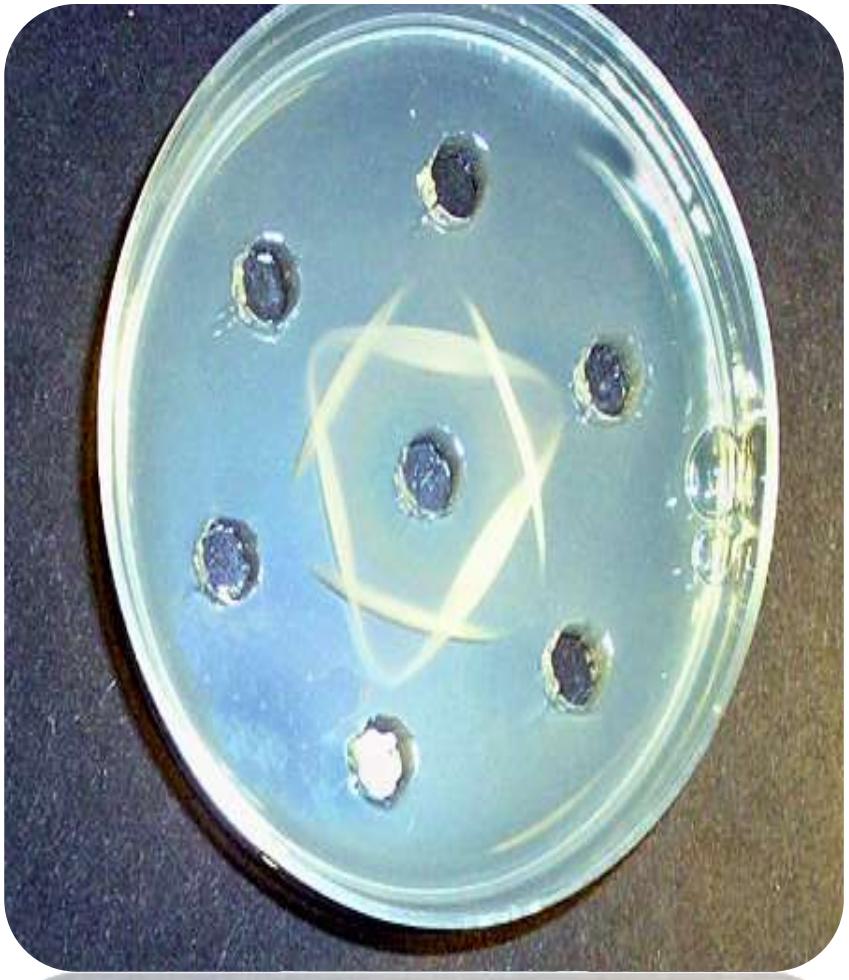


Precipitation Curve



Precipitation Reactions

- Ag-Ab interactions can form visible precipitate
  - Examples:
    - Radial immunodiffusion
    - Double immunodiffusion
    - immunoelectrophoresis



Precipitation

- **Principle**
  - Soluble antigen + antibody (in proper proportions) –> visible precipitate
  - Lattice formation (antigen binds with Fab sites of 2 antibodies)
- **Examples**
  - Double diffusion (Ouchterlony)
  - Single diffusion (radial Immunodiffusion)
  - Immunoelectrophoresis
  - Immunofixation

Precipitation in gels

Based on different rates of diffusion of Ag and Ab into the gel, depending on their :

- concentration
- physicochemical properties
- gel structure

Most widely used gels – agar a agarose

Tests are performed by pouring molten agar (agarose) onto glass slides

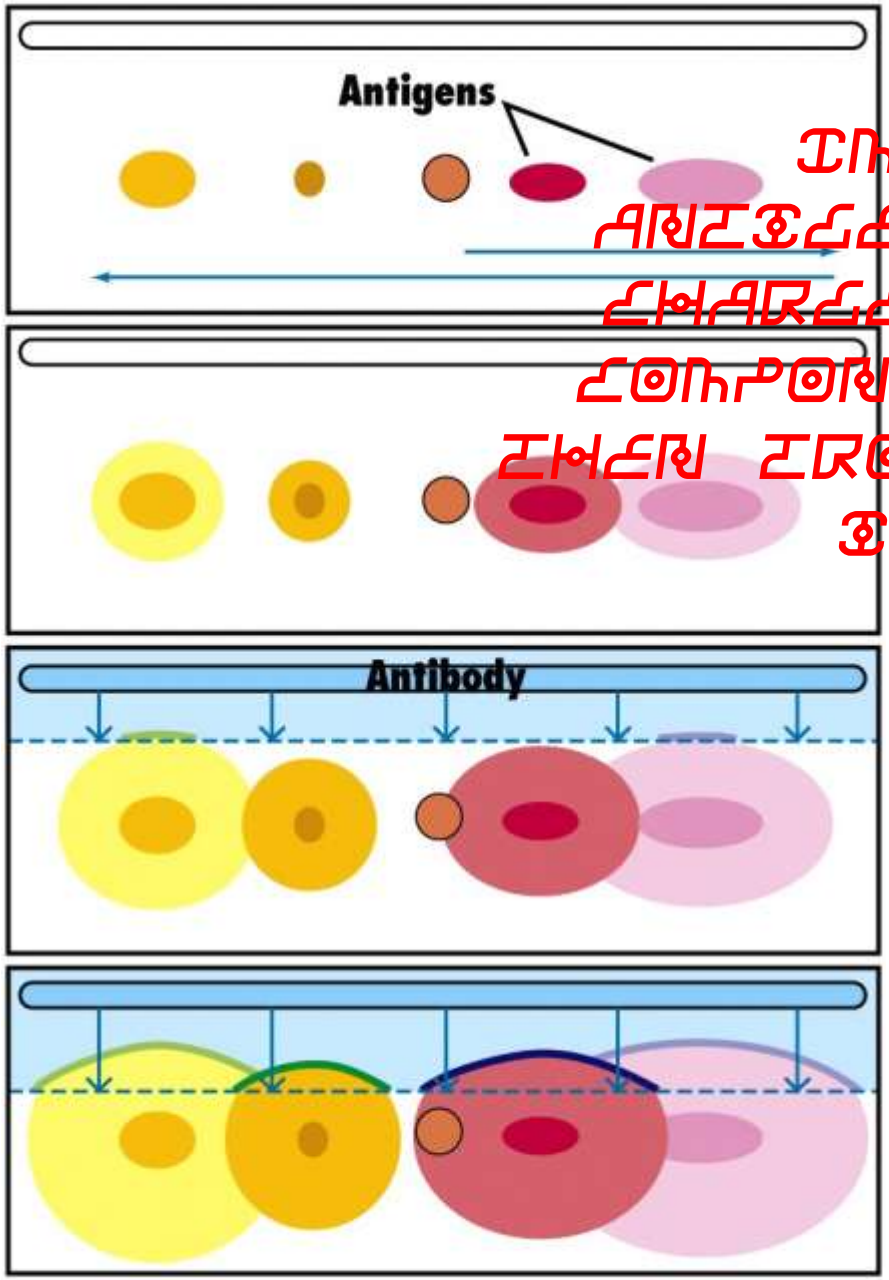
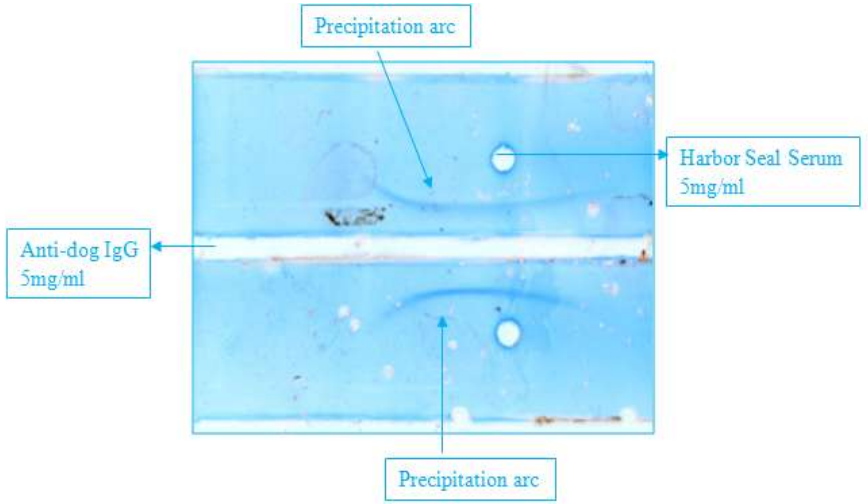


Figure 6-7
Kuby IMMUNOLOGY, Sixth Edition

IMMUNOELECTROPHORESIS
ANTIGEN IS 1ST PUT INTO
CHARGE IS APPLIED TO SEP
COMPONENTS OF ANTIGEN MIX
THEN TROUGHS ARE CUT AND A
IS ALLOWED TO DIFFUSE
THROUGH GEL



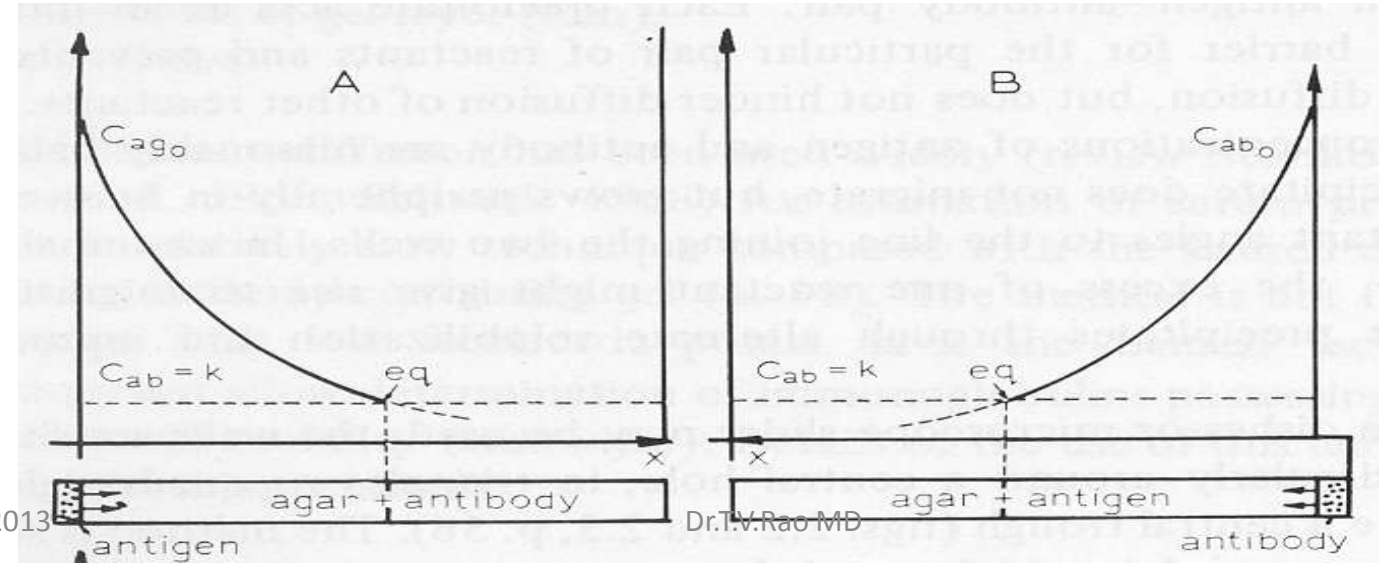
Grabar-Williams Immunoelectrophoresis demonstrating cross-reactivity between Anti-dog IgG and harbor seal serum proteins

Precipitation and Immunodiffusion in gels

Single (simple) diffusion in one dimension:

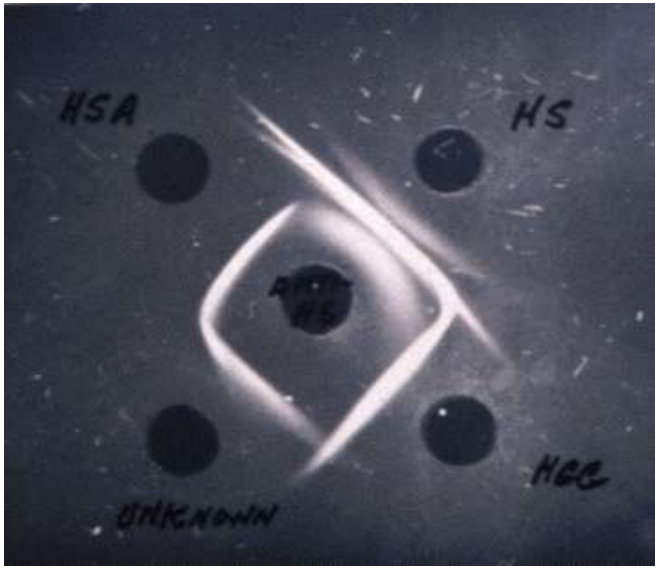
- the process of diffusion of an antigen in an antibody-containing gel
- the process of diffusion of an antibody in an antigen-containing gel.

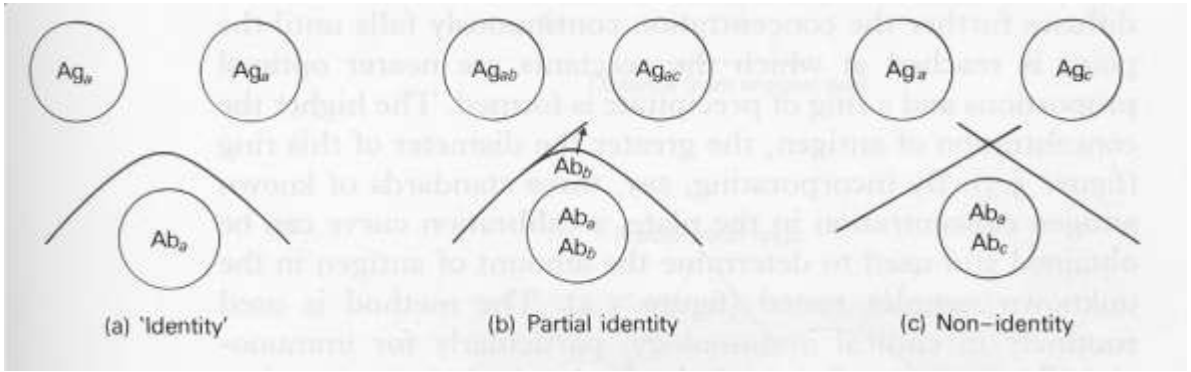
Immunoprecipitin line is formed at the point of equivalence.



Precipitation and Immunodiffusion in Gels

Double diffusion in two dimension





Similar precipitin lines

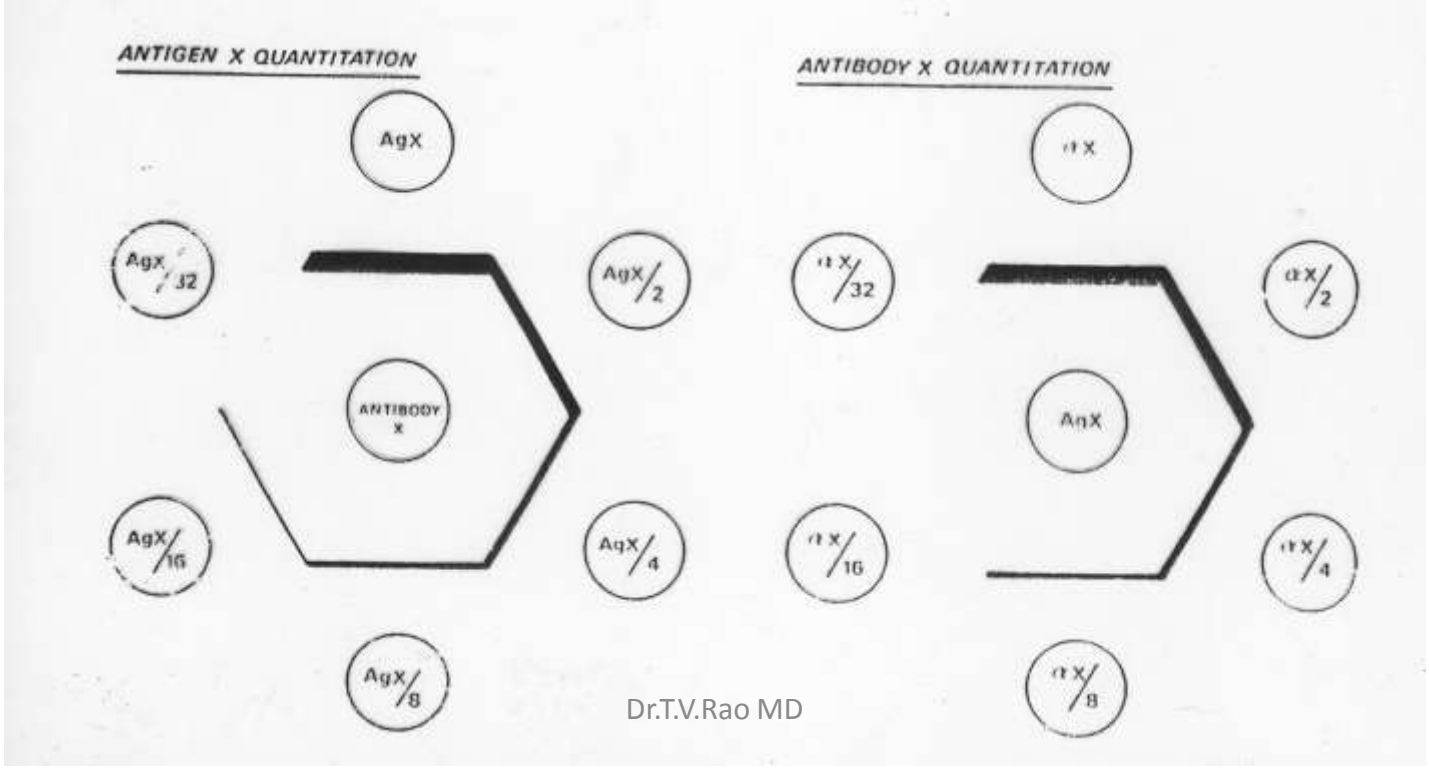
Precipitin lines do not form a complete cross

Precipitin lines completely cross

Precipitation and Immunodiffusion in gels

- **Semiquantitative analysis of:**

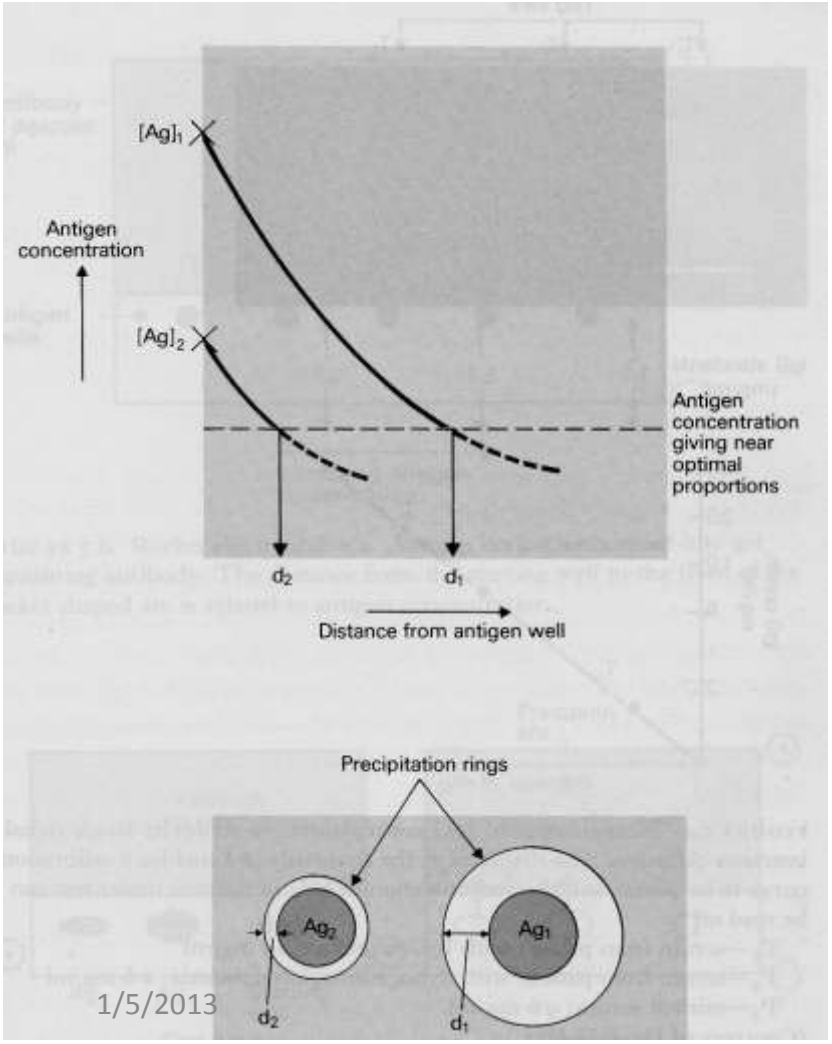
antigen antibody



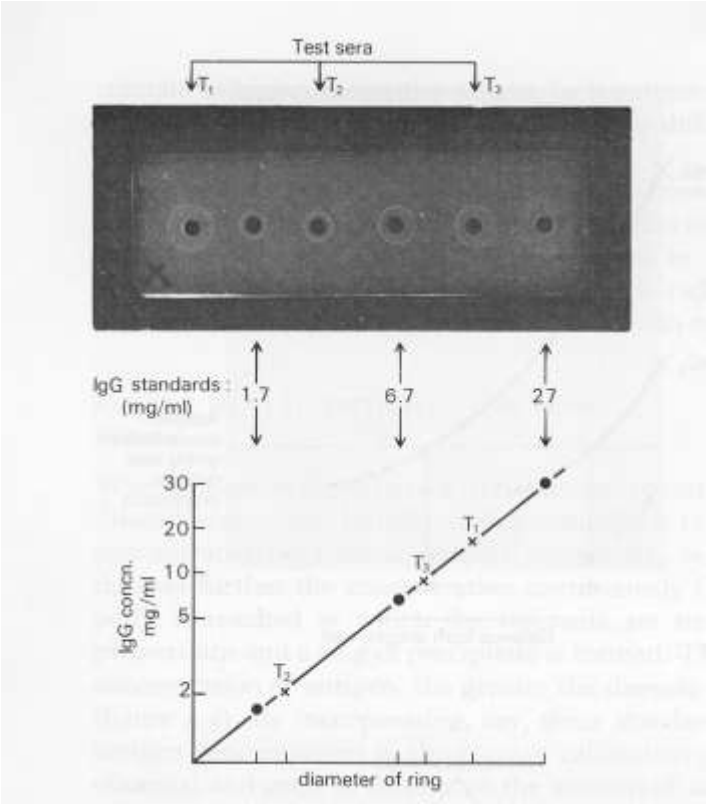
Precipitation and immunodiffusion in gels

- Double diffusion is utilized as a rough estimation of antigen or antibody purity.
- Double diffusion in agar can be used for semi quantitative analysis in human serological system.

Precipitation and Immunodiffusion in gels



Single radial diffusion – quantitative analysis



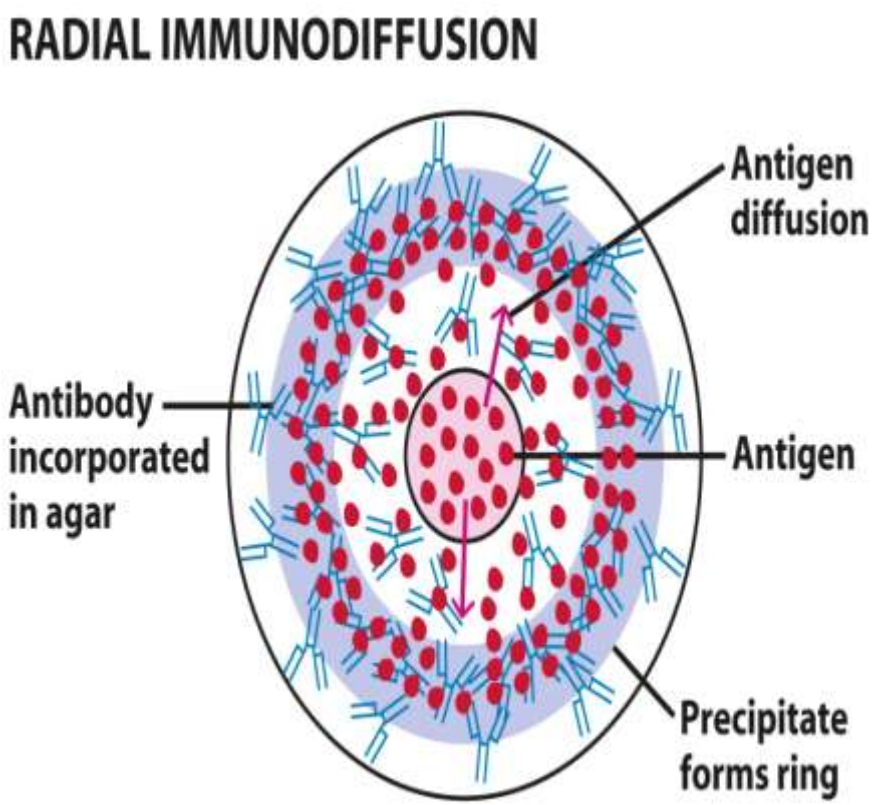


Figure 6-6 part 1
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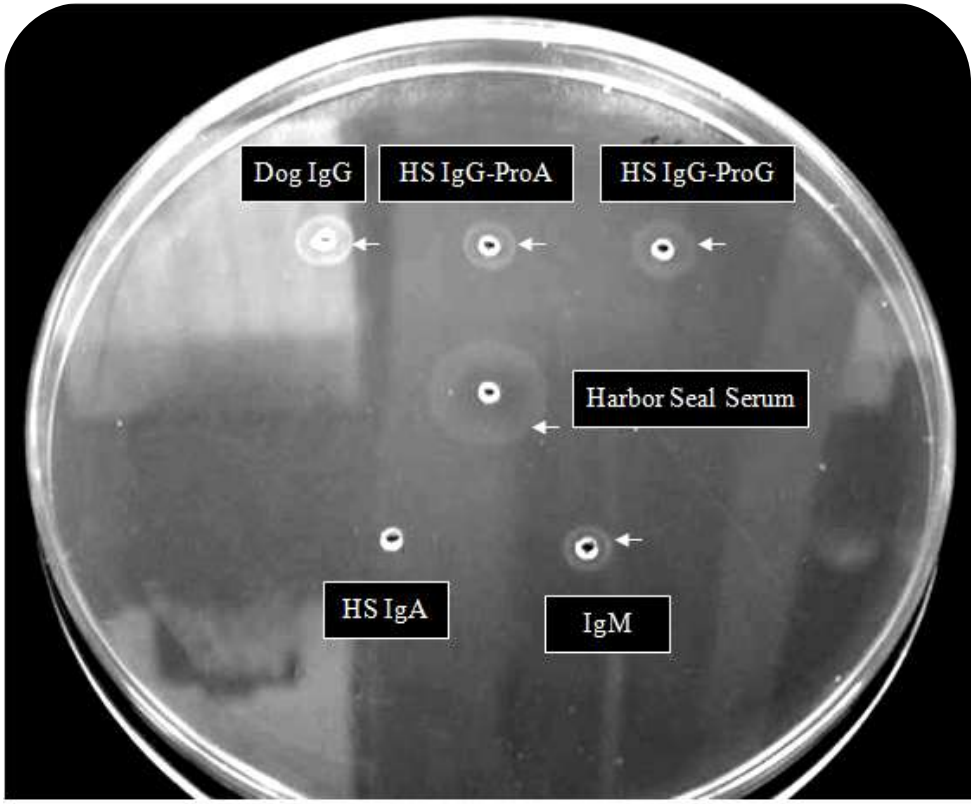


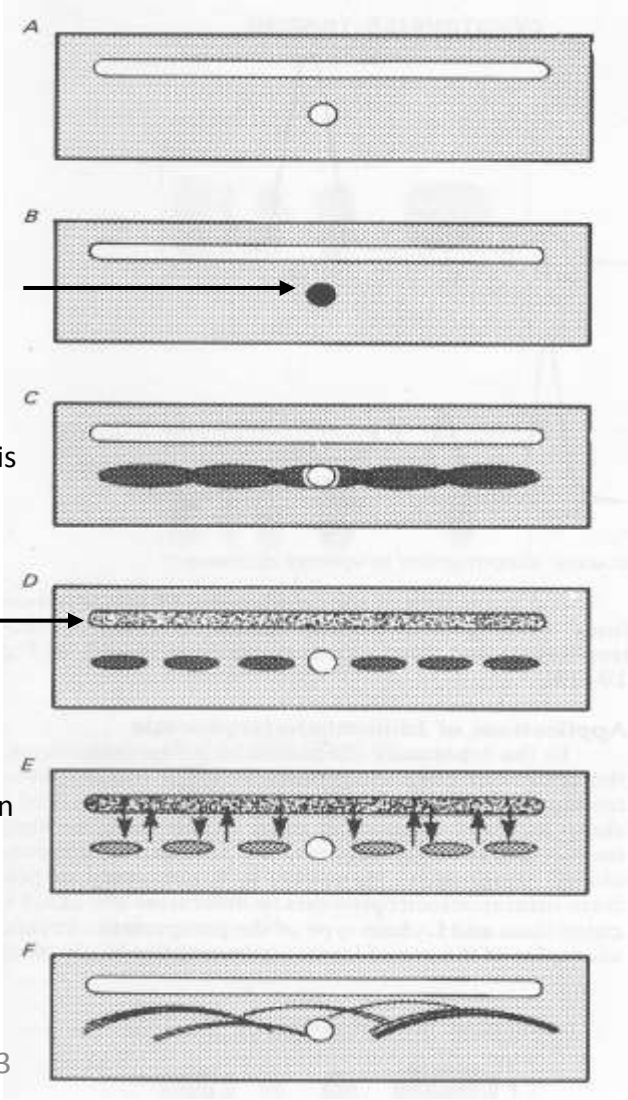
Radial Immunodiffusion representing cross-reactivity of anti-dog IgG with habor seal immunoglobulins

THE FIRST EXAMPLE, ANTI-DOG IGG IS

INDEED THE ONLY WHAT IS

Immunoelectrophoresis

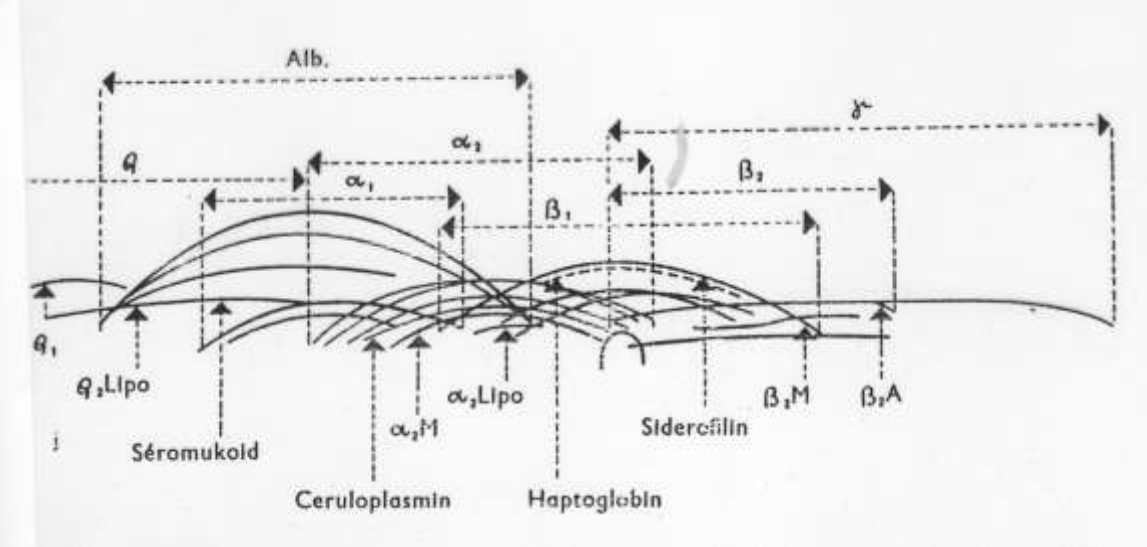
Precipitation and Immunodiffusion in gels

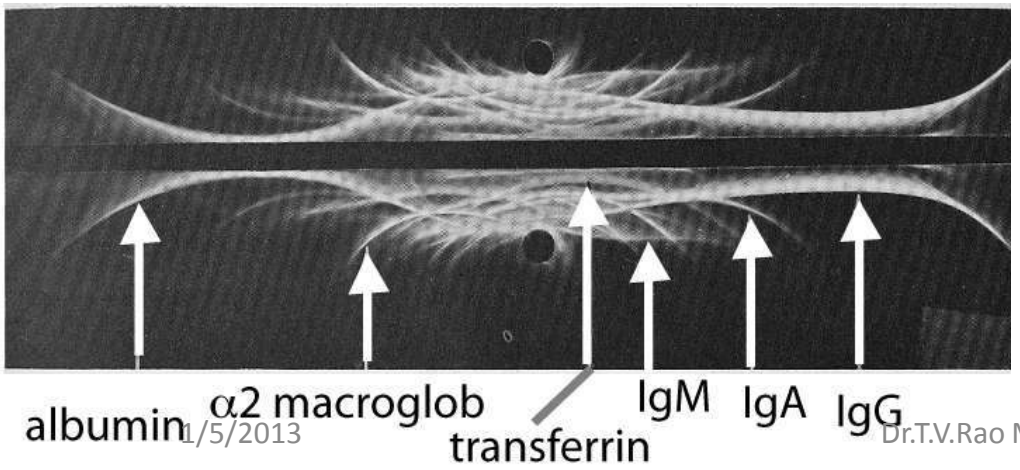


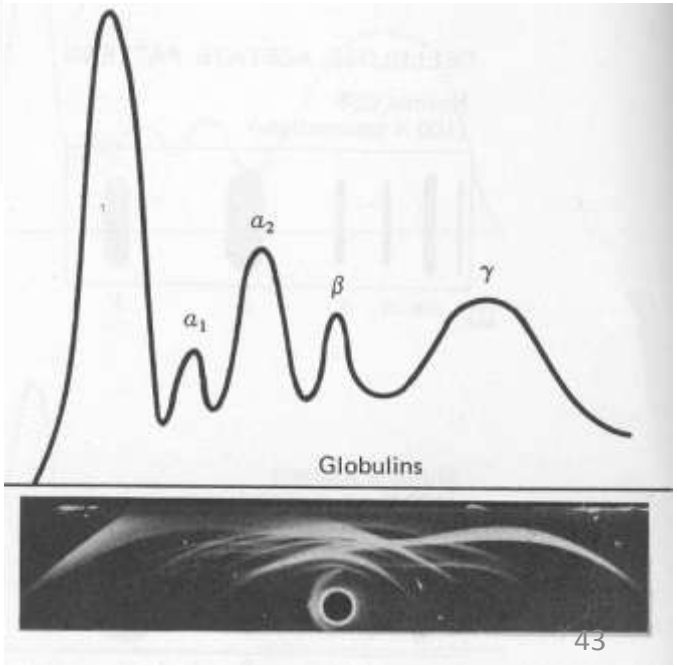
Immunoelectrophoresis

combines electrophoresis separation, diffusion and precipitation of proteins.

Precipitation and Immunodiffusion in gels







Antigen and Antibody reactions can be identified by different methods

Precipitation test

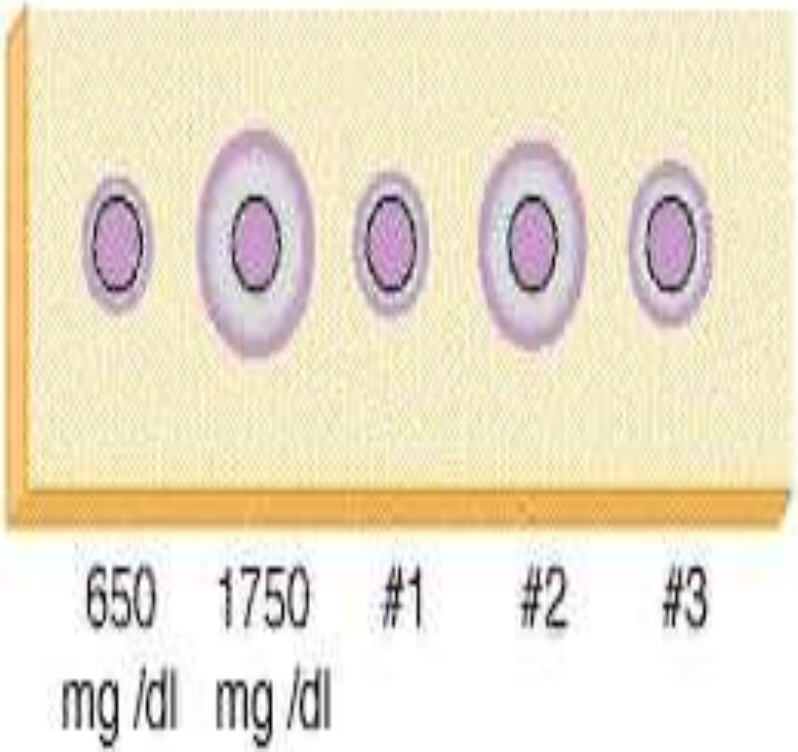
Radial Immunodiffusion (Mancini) –

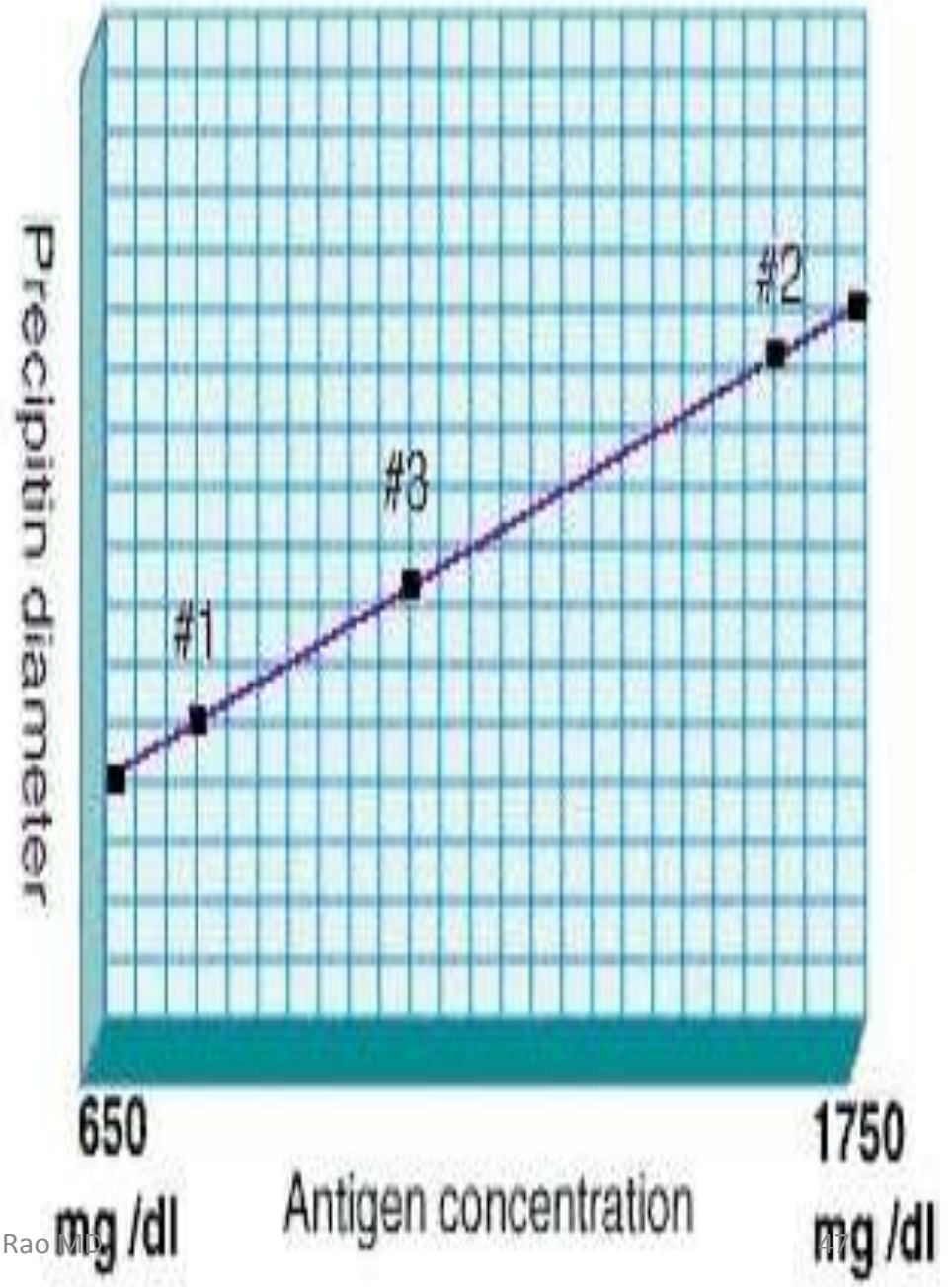
- In radial Immunodiffusion Antigen (IgG antibody) is incorporated into the agar gel as it is poured
- different dilutions of the antibody are placed in holes punched into the agar.
- As the antibody diffuses into the gel it reacts with the antigen and when the equivalence point is reached a ring of precipitation is formed as illustrated in Figure
- The diameter of the ring is proportional to the concentration of antibody since the amount of antigen is constant.

Radial Immunodiffusion (Mancini)

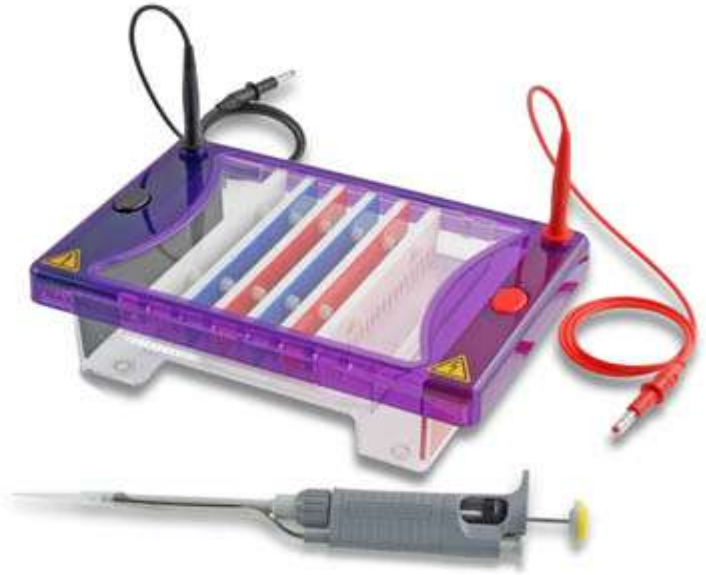
- Thus, by running different concentrations of a standard antibody one can generate a standard cure from which one can quantitate the amount of an antibody in an unknown sample.
- Thus, this is a quantitative test.
- If more than one ring appears in the test, more than one antigen/antibody reaction has occurred. This could be due to a mixture of antigens or antibodies.
- This test is commonly used in the clinical laboratory for the determination of immunoglobulin levels in patient samples.

Radial Immunodiffusion

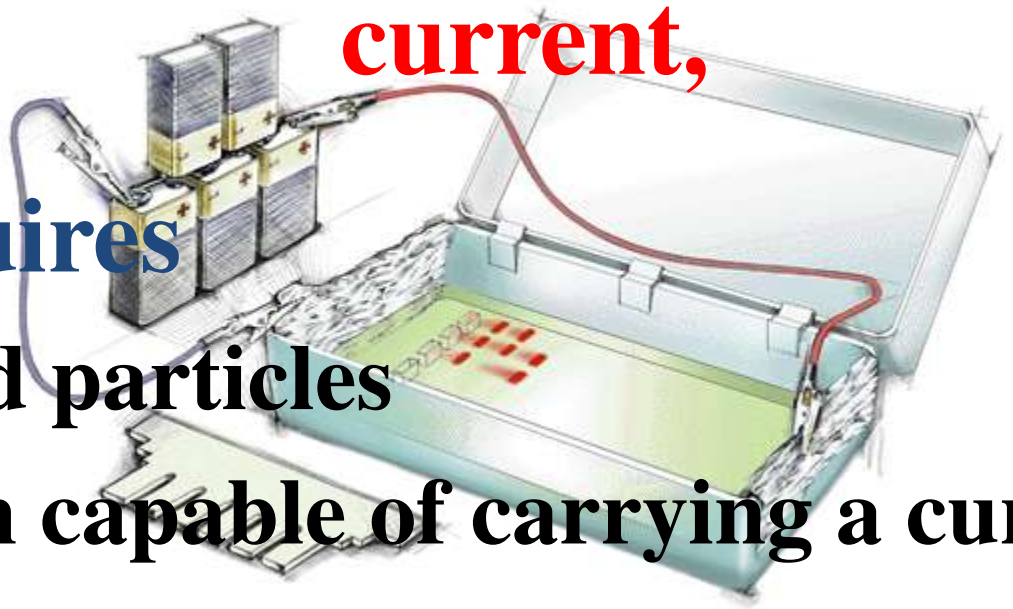




Electrophoresis



The migration of particles under the influence of a direct electrical current,



and requires

- **Charged particles**
- **Medium capable of carrying a current**

What we Need for electrophoresis

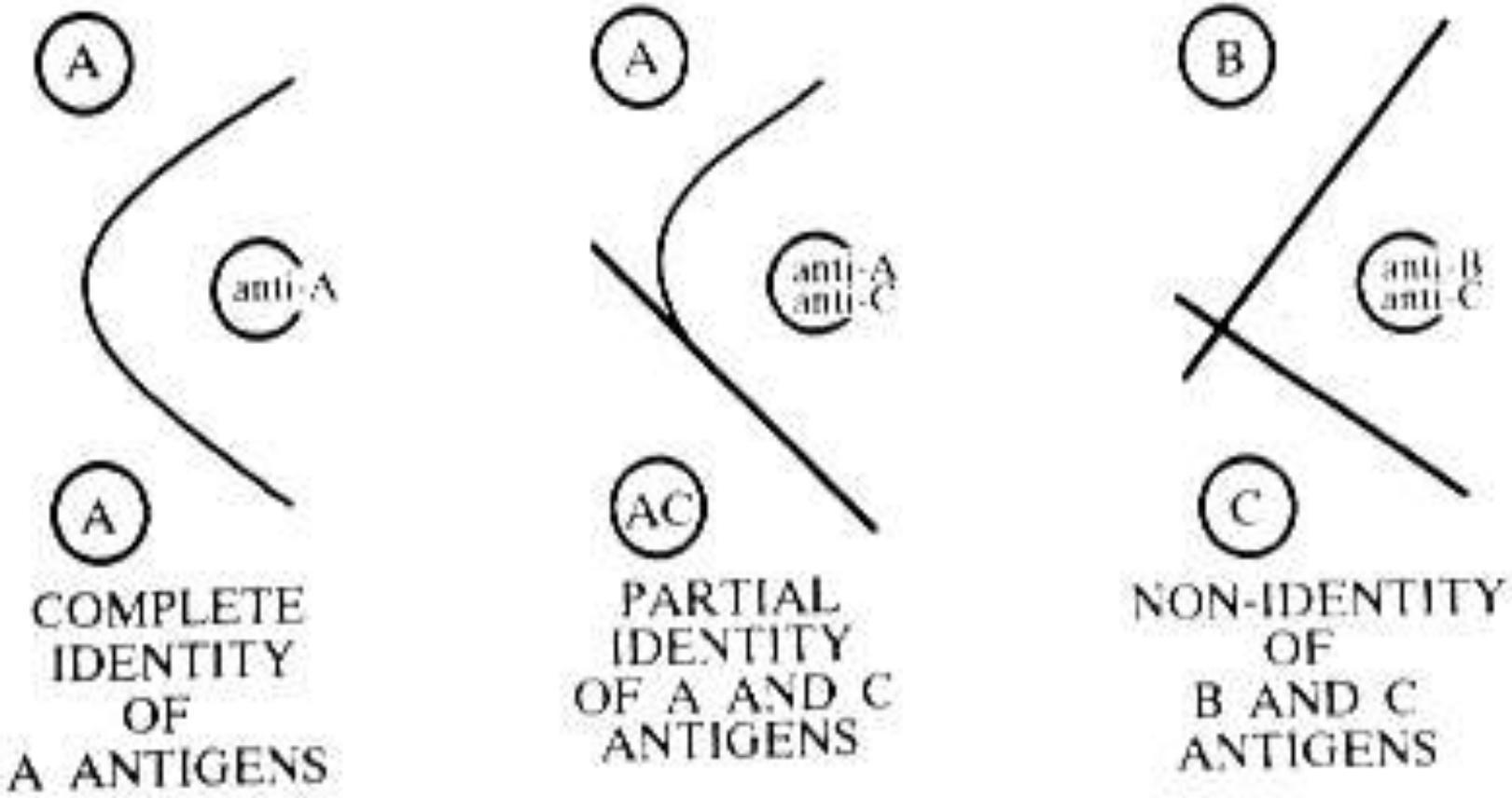
- Choice of electrolyte
  - pH
  - Ionic strength
- Electrophoretic conditions
- Voltage
- Current
- Heat
- Choice of support

Advantages of Electrophoresis

- Ease of use
- Properties affecting separation
- Suitability for appropriate stains or other detection
- Suitability for post electrophoretic data analysis

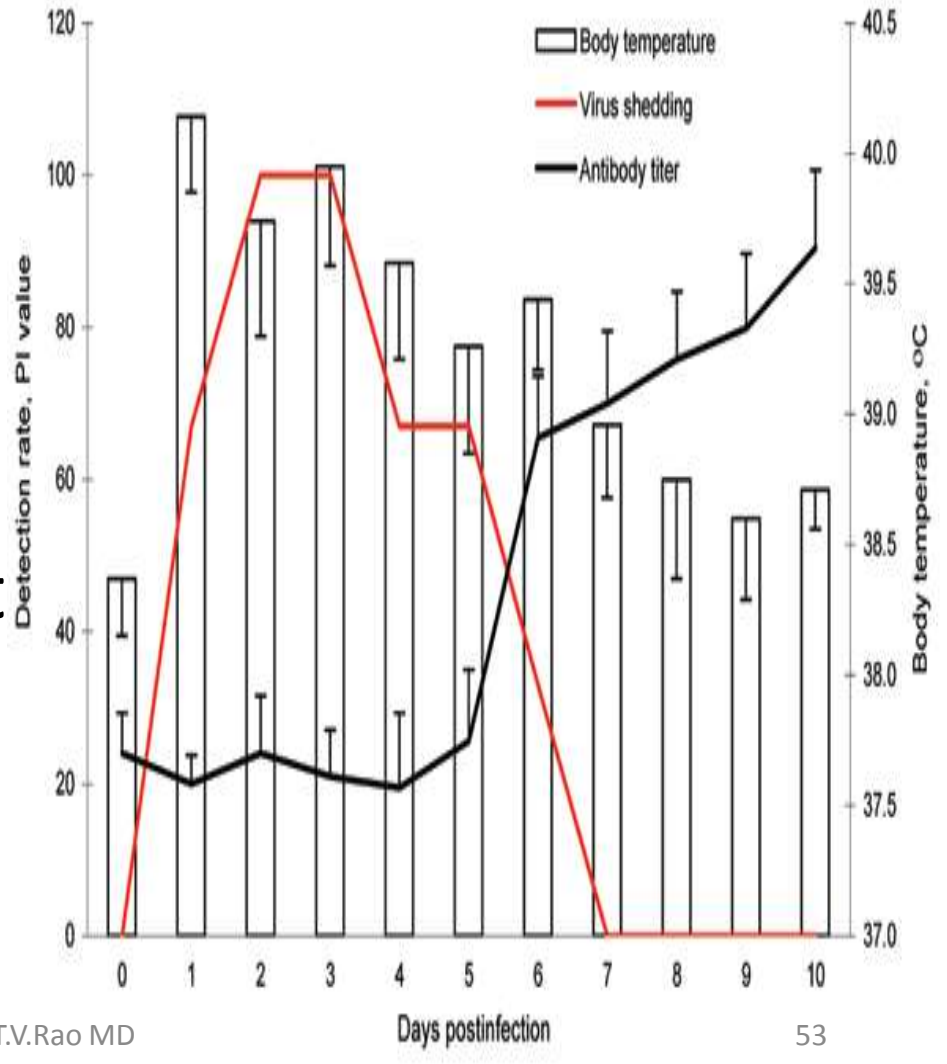
Immunodiffusion

Identification

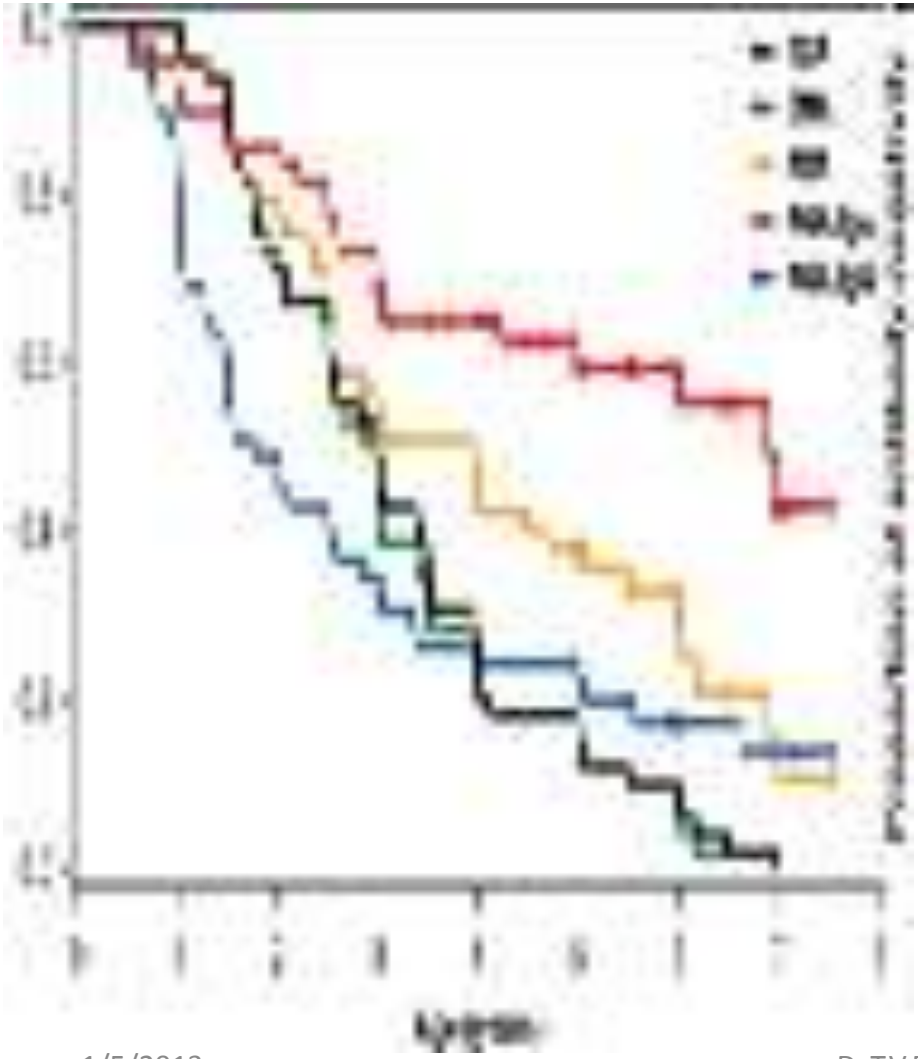


Sero Conversion

- Seroconversion is the development of detectable specific antibodies to microorganisms in the blood serum as a result of infection or immunization.



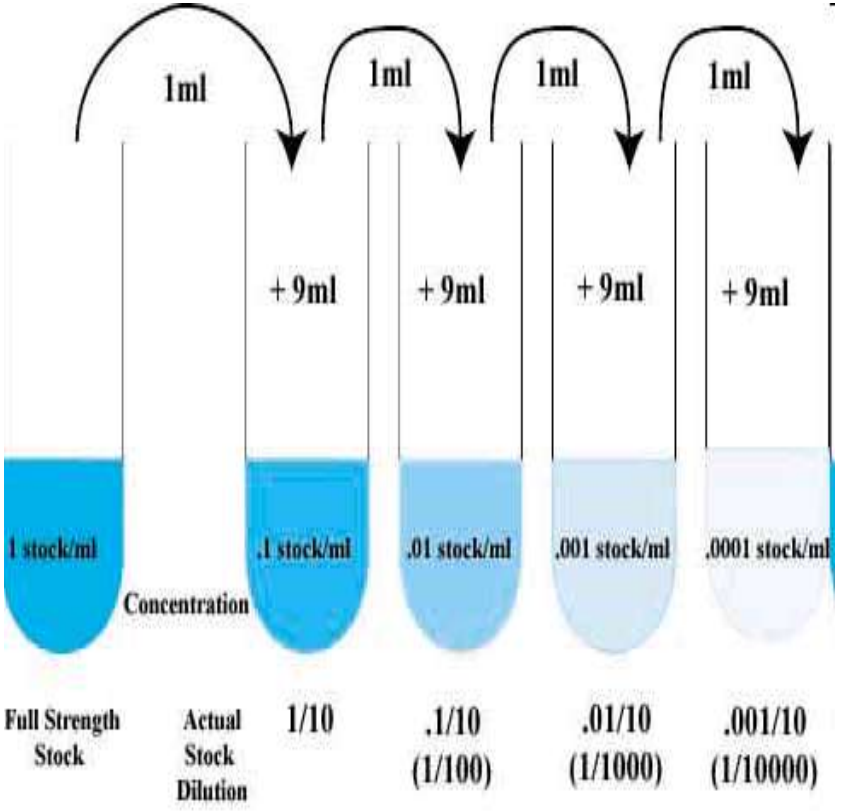
Sero reversion



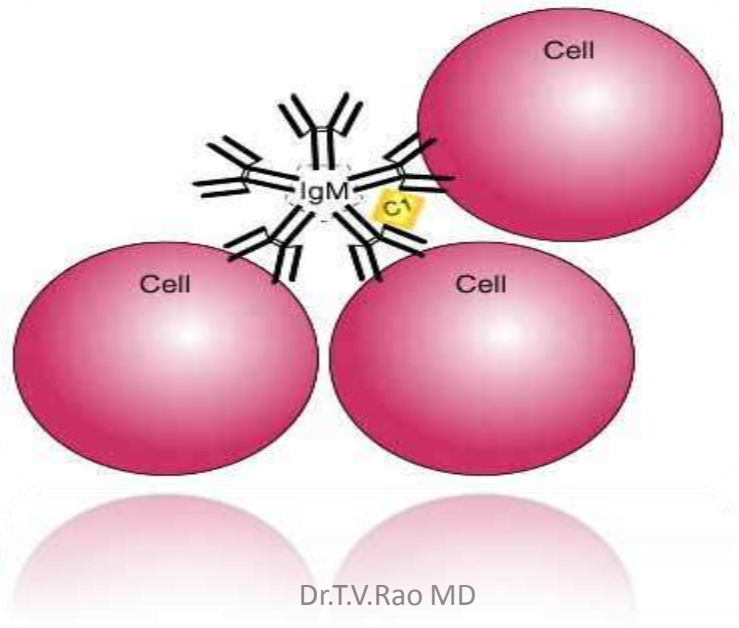
- **Seroreversion** is the opposite of seroconversion. This is when the tests can no longer detect antibodies or antigens in a patient's serum

Testing paired Samples

- Testing for infectious diseases is performed on acute and convalescent specimens (about 2 weeks apart) *Paired sample.*
- **Must see 4-fold or 2-tube rise in titre to be clinically significant**

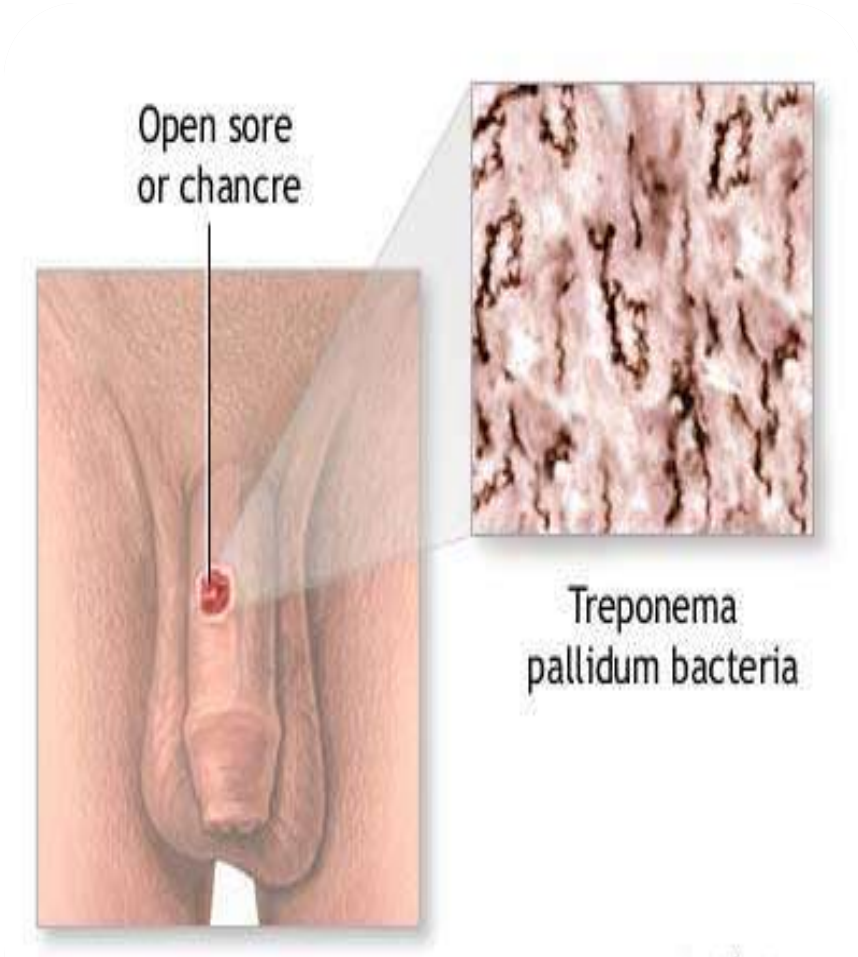


Antigen – Antibody Reactions presenting with precipitation

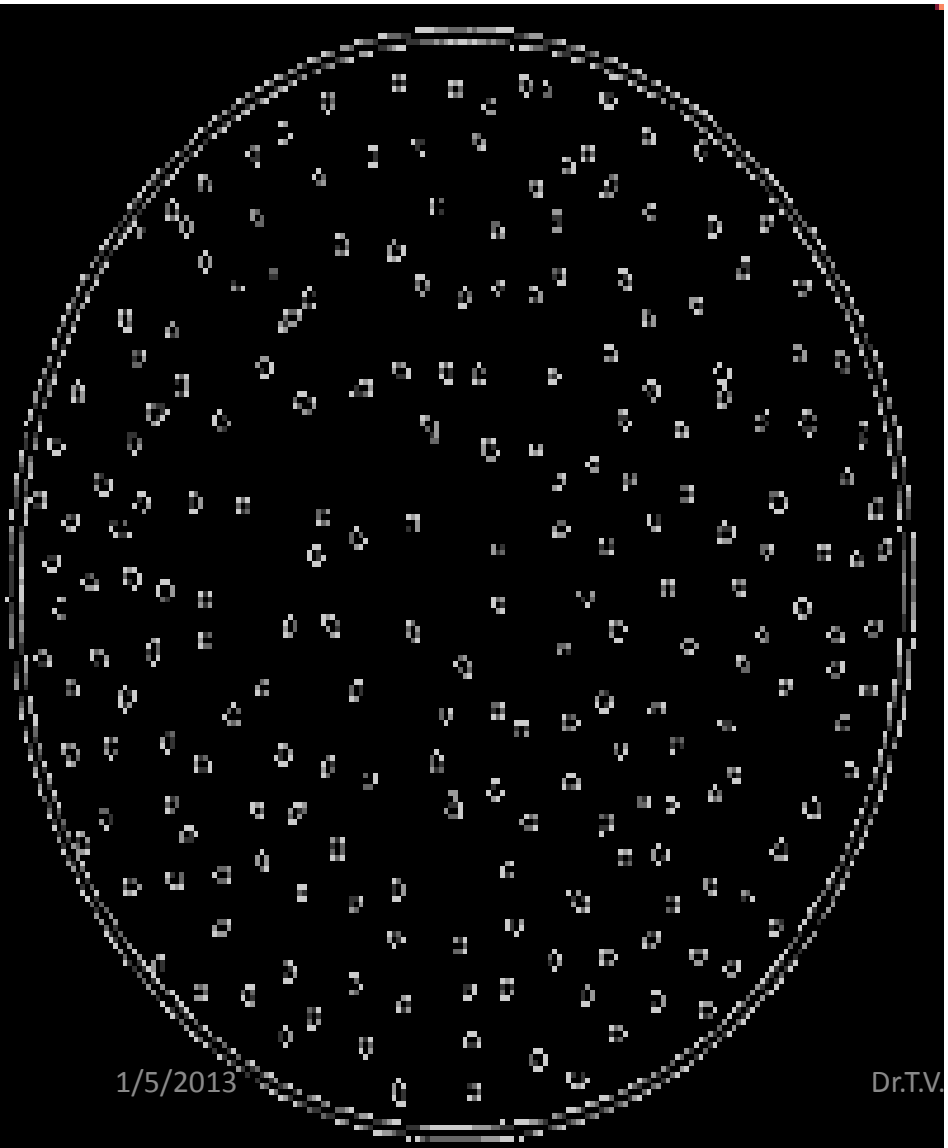


Screening Tests for Syphilis

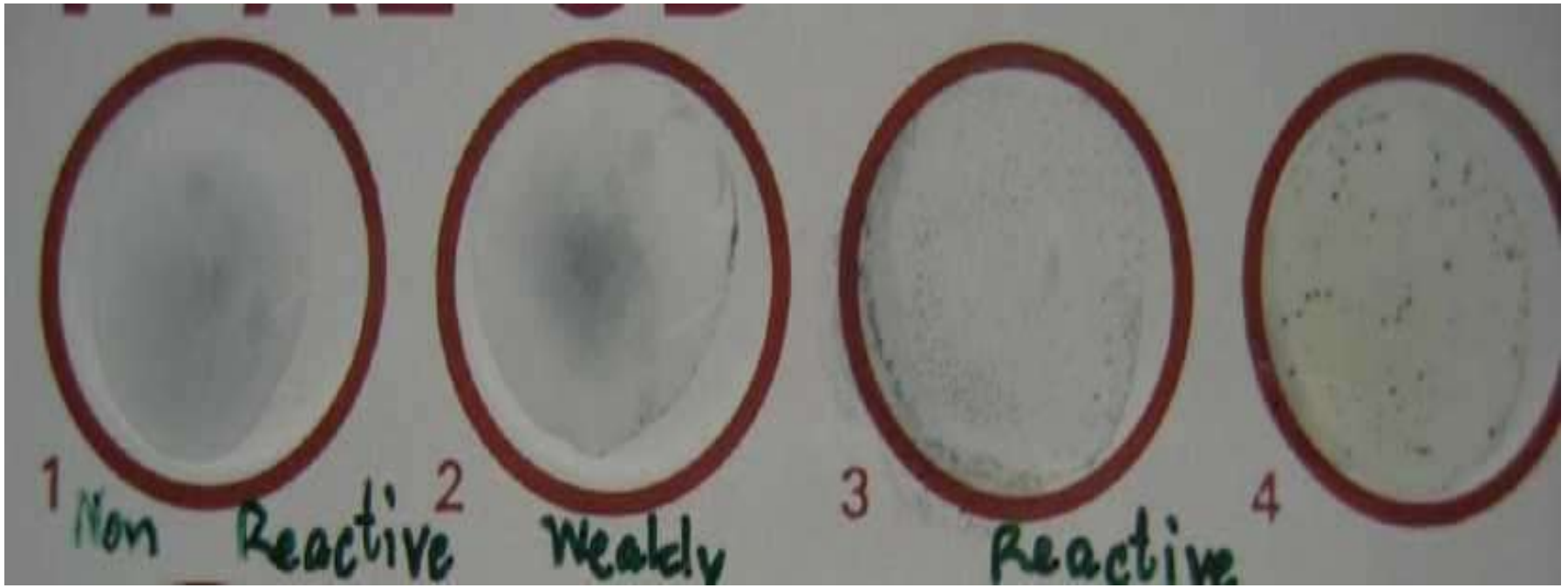
- Serologic methods are divided into two classes. One class, the nontreponemal tests, detects antibodies to lipoidal antigens present in either the host or T. palladium; examples are the **Venereal Disease Research Laboratory and rapid plasma reagin and tests.**



Non reactive and Reactive VDRL Tests



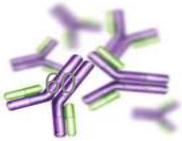
Flocculation test
(A precipitation reaction)



(1) Non Reactive (2) Weakly Reactive (3,4) Reactive

Measurement of Precipitation by Light

- Antigen-antibody complexes, when formed at a high rate, will precipitate out of a solution resulting in a turbid or cloudy appearance.
- Turbidimetry measures the turbidity or cloudiness of a solution by measuring amount of light directly passing through a solution.
- Nephelometry indirect measurement, measures amount of light scattered by the antigen-antibody complexes.



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Immobilization of protease in biopolymers (mixture of alginate-chitosan)

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Immobilization of protease in biopolymers (mixture of alginate-chitosan)

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ABSTRACT

The Protease enzyme breaks down protein into amino acids as its constructors. Protease immobilization onto appropriate support materials plays an essential role in various fields of technology including the food and detergent industries. Accordingly, improvement of protease immobilization has been highly regarded due to its applications in bio catalysis. Alginate and chitosan are natural polysaccharides that have been studied so extensively in enzyme immobilization.

In this research, the physical immobilization of proteases in alginate-chitosan beads showed satisfactory activity and stability. These beads were prepared by adding protease-alginate dropping into Chitosan and Calcium chloride solution. Then proteases enzyme encapsulated in alginate-chitosan beads. In the end, the different conditions such as temperature, pH and stability of the enzyme were studied.

The immobilized protease was optimized in temperature of 47°C and at pH 8.5. The results demonstrated that the protease enzyme immobilized in alginate-chitosan beads exhibits reasonable stability and good activity.

Keywords: Protease enzyme; Biopolymer; Alginate; Chitosan; Immobilization; Stability.

INTRODUCTION

Enzymes are biological catalysts that facilitate the conversion of substrates into products by providing favorable conditions that lower the activation energy of the reaction. An enzyme may be a protein or a glycoprotein and consists of at least one polypeptide moiety [1]. Serine alkaline proteases (SAP) are one of the most important groups of industrial enzymes that are widely used in detergent, leather and meat industries. They account for approximately 35% of the microbial enzyme sales [2]. Immobilization is a general term describing a wide variety of the cell or particle attachment or entrapment in spherical beads [3]. It can be applied to basically all types of biocatalysts including enzymes, cellular organelles , animal and plant cells. Currently, different kinds of immobilization techniques have wide applications not only in the field of biotechnology, but also in pharmaceutical, environmental, food and biosensor industries [4]. Their use as detergent additives still represents the largest application of industrial enzymes, both in terms of volume and value[5]. All detergents contain similar ingredients and are based on similar detergency mechanisms. To improve detergency, modern types of heavy-duty powder detergents and automatic dishwasher detergents usually contain one or more enzymes [6]. Immobilization is generally necessary for optimum performance in non-aqueous media. In the traditional method of using enzymes as lyophilized (freeze-dried) powders, many of the enzyme molecules are not readily accessible to substrate molecules [7]. Immobilization can also help to enable the use of enzymes in different conditions such as chemical solvents, pH, temperature and exceptionally high substrate concentrations [8]. Covalent binding is a conventional method for immobilization; it can be achieved by direct attachment with the enzyme and the material through the covalent linkage. Covalent method of immobilization is mainly used when a reaction process does not require enzyme in the product, this is the criteria to choose covalent immobilization method [9]. Adsorption involves the physical binding of enzymes on the surface of an inert support [10].

Alginate is a natural polysaccharide that is synthesized by brown seaweeds and by soil

bacteria [11]. It is widely employed in the food processing industry [12]. Sodium alginate is the most commonly used alginate form in the industry, since it is the first byproduct of algal purification. Sodium alginate consists of α-L-guluronic acid residues (G blocks) and ß-D-mannuronic acid residues (M blocks), as well as segments of alternating guluronic and mannuronic acids (GM blocks) [13].

The calcium alginate matrix formed is usually very permeable and little or no drug release can actually be controlled in the case of soluble drugs [14]. Chitosan is a natural based-polymer obtained by alkaline deacetylation of chitin. This biopolymer is nontoxic, biocompatible, and biodegradable. These properties make chitosan a good candidate for the development of conventional and novel drug delivery systems. Chitosan has been found to be used as a support material for gene delivery, cell culture, and tissue engineering. However, practical use of chitosan has been mainly confined to the unmodified forms [15].

The present research was based on the entrapment of *Bacillus subtilisin calsberg* in calcium alginate-chitosan- mixture and the characteristics of immobilized enzyme such as activity and stability were assessed.

MATERIALS AND METHODS

Protease assay

Protease was purchased from sigma. Activity of protease enzyme was determined by Anson method [16]. In this method, 5.4 mg of enzyme was added in 1ml Tris-HCL buffer (pH=8.1) and mixed with 0.5 ml of casein solution.

The solution was incubated in a water bath at 37°C for 20-30 minutes. After that TCA (110mM) was added to stop the reaction. Finally the solution was mixed with 5ml of sodium carbonate and 0.5 ml of Folin's reagent for 25 minutes and the absorbance value was determined in 660nm.

Preparation of Alginic acid-Protease Enzyme Solution

First of all 200 mg of alginate was dissolved in 10 ml Tris-HCl buffer (0.1M pH 8.0) by heating at 30°C-40°C for 30 minutes. When the dissolving of all alginate particles completed, the liquid protease enzyme (0.5 mg/ml) was slowly added by stirring into the alginate solution. This mixture was stirred for 1 hour for complete homogenate of enzyme and alginate.

Preparation of Chitosan/CaCl2 Solution

500 mg of chitosan was added to acetic acid media (v/v, 2%) by heating at 50°C.

After gained a clear solution, $CaCl_2$(0.7 M) was added to the chitosan solution and was stirred at 50°C to 55°C.

Production of enzyme entrapped Alginate-Chitosan beads

In this study two phases were prepared. One of them is calcium chloride-chitosan and the other one is enzyme mixed with alginate solution. The protease-alginate mixture was added into chitosan-calcium chloride by means of a syringe.

Assay of immobilized protease enzyme

5.4 mg of immobilized enzyme was added in 1ml Tris-HCL buffer (pH=8.1) and mixed with 0.5 ml of casein solution. The solution was incubated in a water bath at 37°C for 30 minutes. After that TCA (110mM) was added to stop the reaction. The mixture was filtered by means of a filter paper. Finally the solution was mixed with 5ml of sodium carbonate and 0.5 ml of Folin's reagent for 25 minutes and the absorbance value was determined in 660nm.

Effect of different temperatures on immobilized protease enzyme

The immobilized protease activity was illustrated at different temperatures ranging from 20°C to 57°C in Anson method.

Scanning Electron microscope

The surface morphology of the immobilized enzyme in calcium alginate-chitosan beads are examined using a scanning electron microscope (LEO 44I).

Effect of pH on immobilized protease enzyme

The immobilized protease activity was considered by different pH values (7, 7.5, 8.5, 9 and 9.5) in Anson method.

RESULTS

In this project we studied the immobilized protease enzyme in various condition such as temperature, pH, stability.

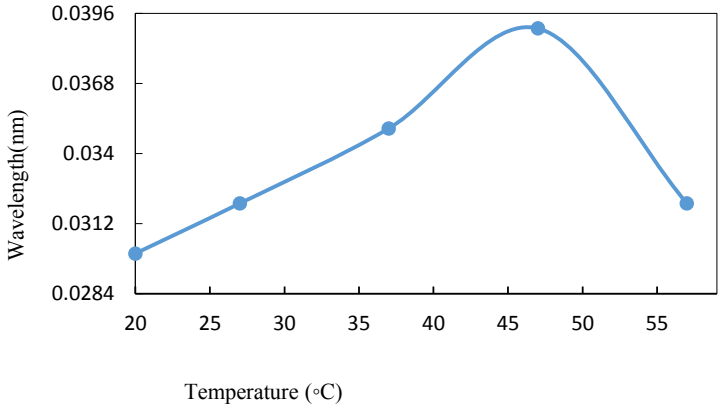

Figure 1. Effect of different temperatures on immobilized protease activity from *Bacillus subtilisin Carlsberg.*

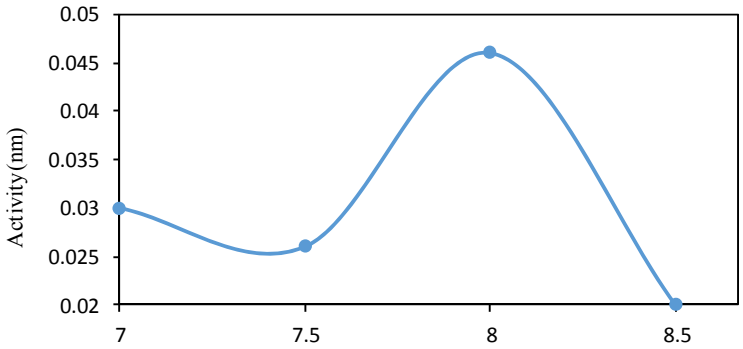

Figure 2. Effect of different pH on immobilized protease activity from *Bacillus Carlsberg.*

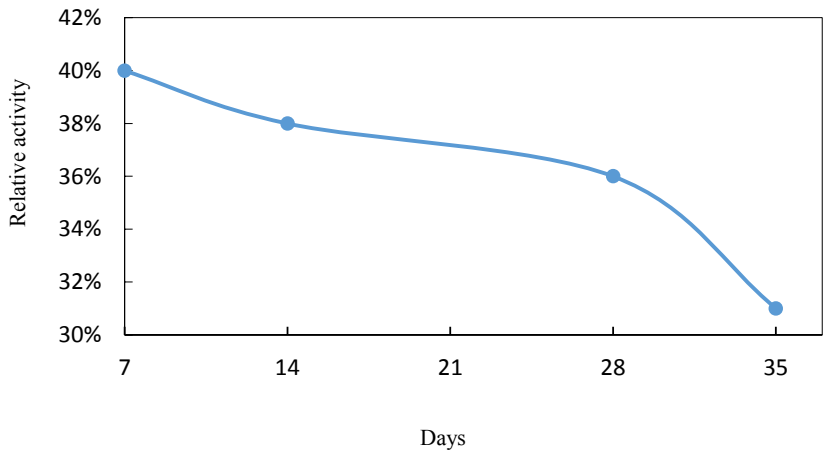

Figure 3. Decrease of immobilized protease activity from *Bacillus subtilisin calsberg* in 35 days.

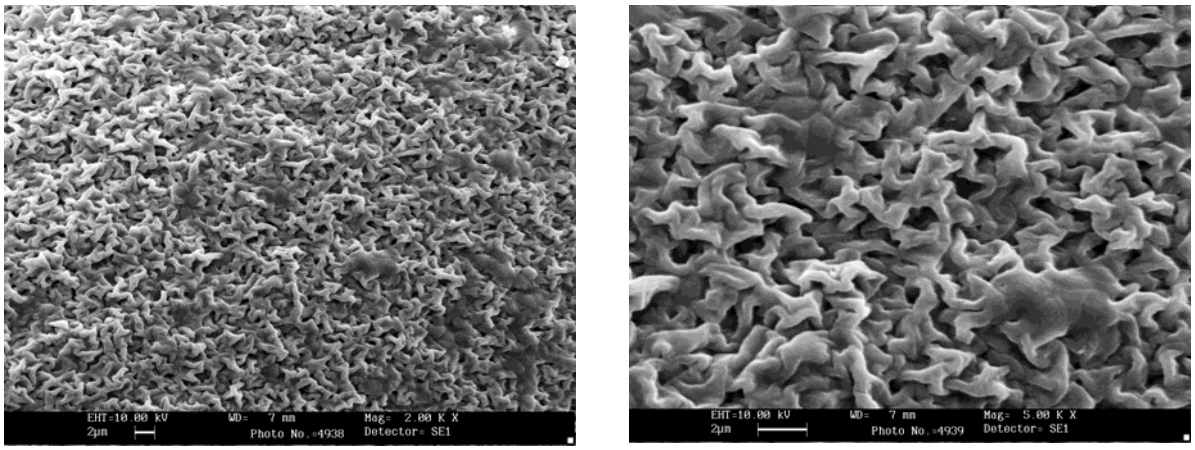

Figure 4. (a) Scanning electron with 2.00 kx mag. micrographs of calcium alginate-chitosan microspheres-Protease.**(b)** Scanning electron with 5.00 kx mag. micrographs of calcium alginate-chitosan microspheres-Protease.

Effect of temperature on stabilized protease enzyme

As it is shown in Figure 1 we reported that optimum temperature was 47°C.

Effect of pH on immobilized protease enzyme

Investigation of pH effect on immobilized enzyme activity was done at different pH values (7 , 7.5, 8.5, 9 and 9.5). The optimum pH was 8.5 (Figure 2).

Stability of encapsulated protease enzyme

The stabilization of immobilized enzyme has been studied in 5 weeks. In one week, the relative activity decreased up to 40%; then gradually, week by week, it mitigated up to 30% (in orderly, in the second week the relative activity reduced up to 36%. The week later, 31% and eventually, in the last week, the relative activity was observed 30%)
(Figure 3).

The immobilized protease enzyme surface morphology

The immobilized enzymes morphology was studied by using SEM (Fig 4a, 4b). The results demonstrated that the protease enzyme has stabilized in calcium alginate-chitosan-mixture has a compact structure.

DISCUSSION

In general, high temperature enhances the rate of an enzyme's activity, because at high temperatures, molecules move around faster, so an enzyme is likely to come in contact with a substrate very quickly. In 2011, the effect of temperature on immobilized Glucose Oxidase in Alginate-Chitosan Microcapsules has been studied [17]. In 2009 some scientists surveyed immobilized protease enzyme in different temperatures [18]. They reported that optimum temperature was 50°C when enzyme entrapped in alginate beads alone (without chitosan). In this investigation, the absorbance value concerning immobilized enzyme activity was recorded in five temperatures from 20°C to 57 °C. It was concluded that the temperature 47°C was identified as the most preferable and appropriate temperature for immobilized enzyme. The activity of enzymes was strongly relied on various pH, and each enzyme met the best activity range at certain pH value. In this study, among of the effects of pH on immobilized enzyme activity, five of them have been analyzed (at pH7, 7.5, 8.5, 9 and 9.5); hence, the most primary pH recognized was at 8.5 (Figure 2); While in 2009, the best pH for protease stabilized on alginate (without chitosan) was reported at 10.5 [10].

In this study we investigated alginate-chitosan surface with SEM .Many large hollow pores or multiple small hollow pockets were observed in the alginate matrix [19].

The size of the beads was measured by Scanning Electron Microscope. The diameter of each bead was measured at three different angles and averaged. 7 beads were used to give an average bead size. The average bead size measured by an optical microscope was 2.6±0.2, 1.8±0.2. The activities of protease entrapped in the beads decreased as the bead size increased all samples were coated with gold prior to observation. The surface of the coated beads has looked like a mesh, and has very compact structures [20].

ACKNOWLEDGEMENT

The authors would like to thank the Islamic Azad University Science and Research branch, Malek-Ashtar university for financial supporting.

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Immune Cells and Organs

Elizabeth Repasky, Sept. 1, 2016

Dept of Immunology
Elizabeth.repasky@roswellpark.org

Immune system Purpose/function?

- First line of defense= epithelial integrity= skin, mucosal surfaces
- Defense against pathogens
  - Inside cells= kill the infected cell (Viruses)
  - Systemic= kill- Bacteria, Fungi, Parasites
- Two phases of response
  - Handle the acute infection, keep it from spreading
  - Prevent future infections

The Immune System

"Although the lymphoid system consists of various separate tissues and organs, it functions as a single entity. This is mainly because its principal cellular constituents, lymphocytes, are intrinsically mobile and continuously recirculate in large number between the blood and the lymph by way of the secondary lymphoid tissues… where antigens and antigen-presenting cells are selectively localized."

-Masayuki, Nat Rev Immuno. May 2004

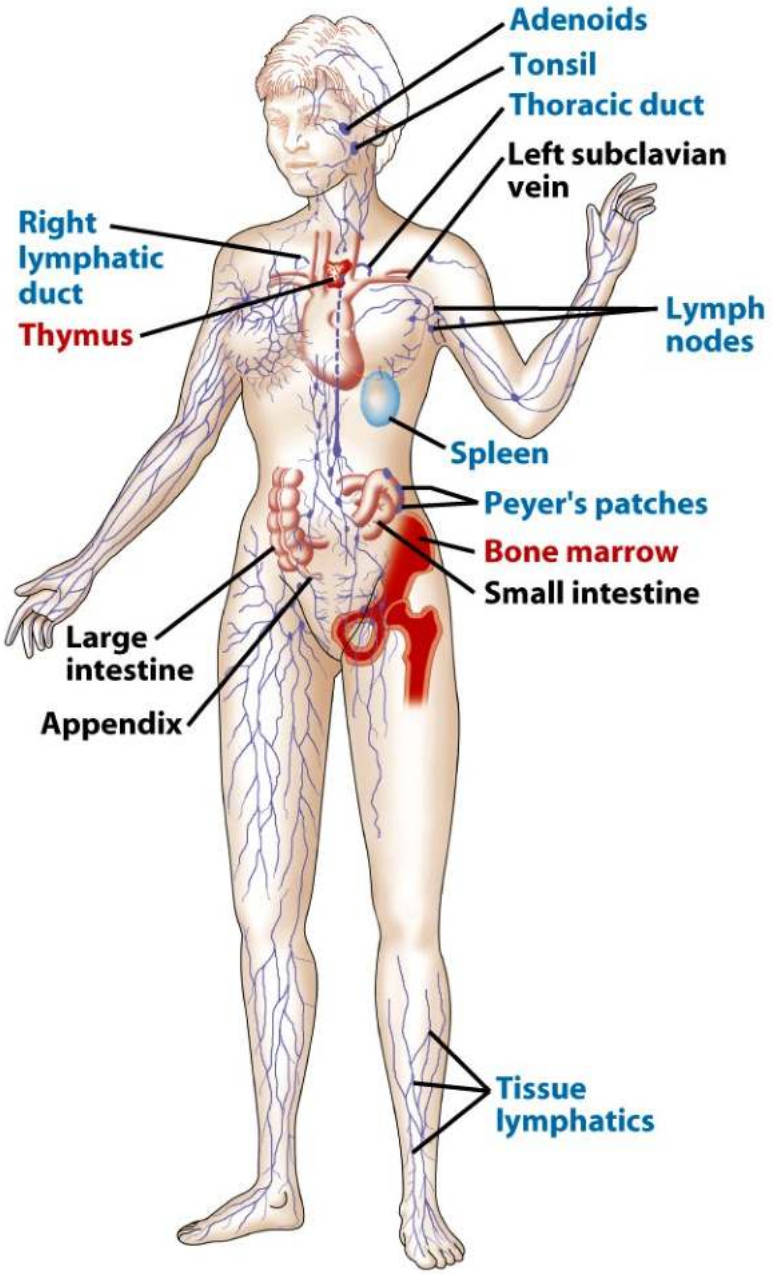
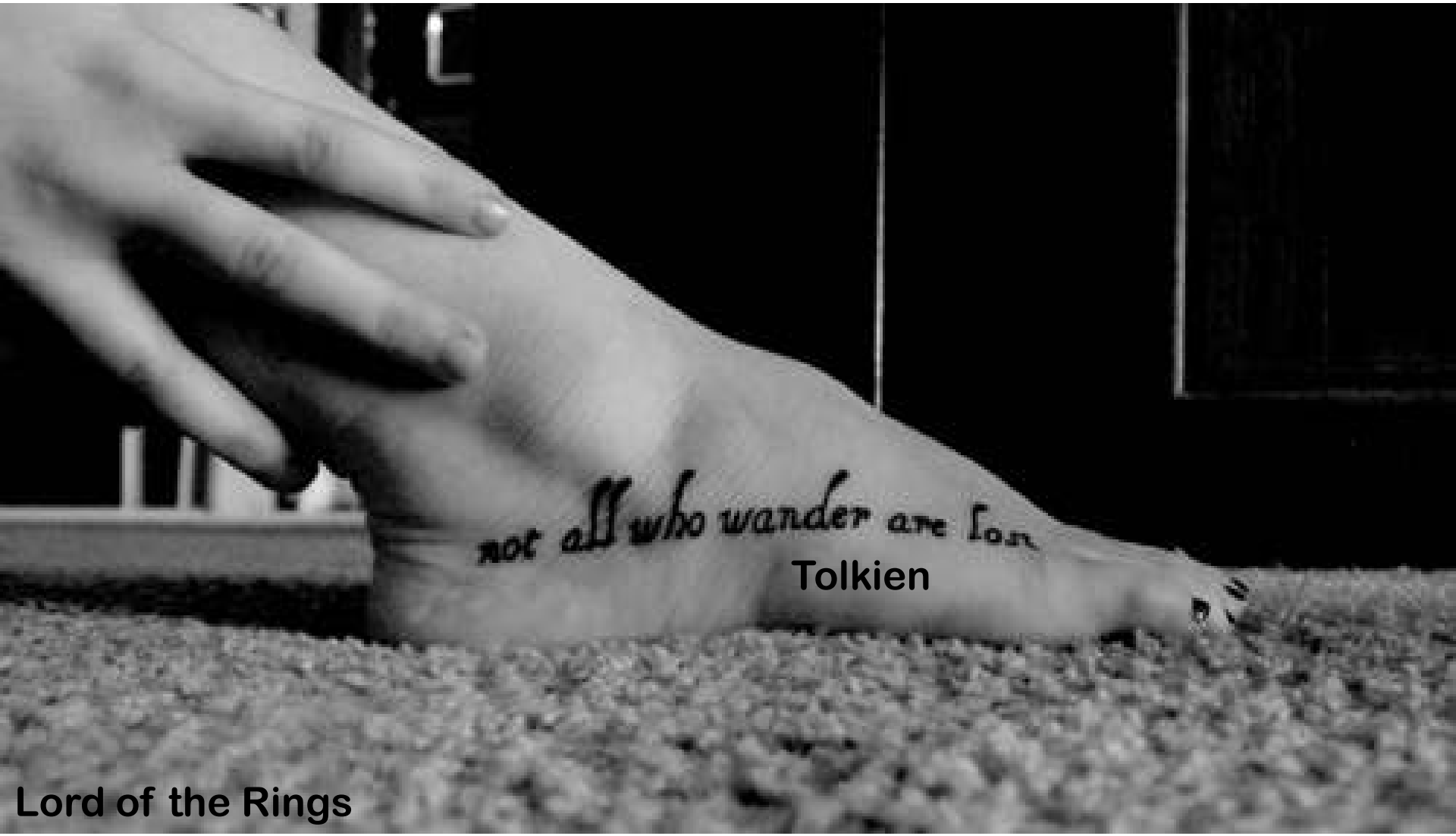


Figure 2-11
Kuby IMMUNOLOGY, Sixth Edition

Not all who wander are lost…..



…..some are searching

Immune System

- Cells
  - Innate response- several cell types
  - Adaptive (specific) response- lymphocytes
- Organs
  - Primary where lymphocytes develop/mature
  - Secondary where mature lymphocytes and antigen presenting cells interact to initiate a specific immune response
- Circulatory system- blood
- Lymphatic system- lymph

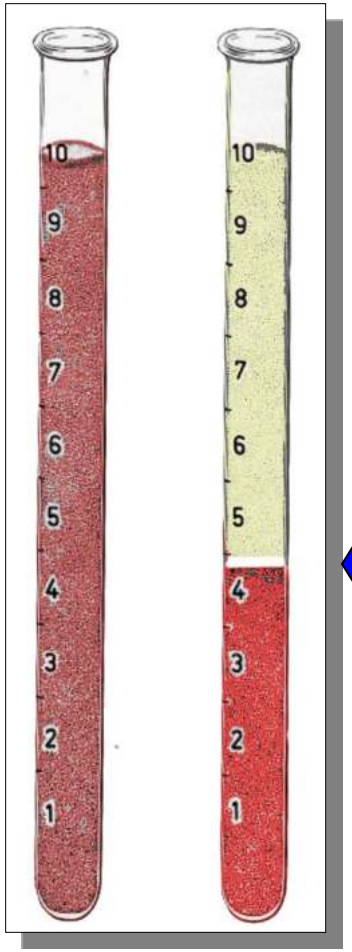
Cells= Leukocytes= white blood cells

Granulocytes

1. neutrophils
2. eosinophils
3. basophils

Non-granulocytes

4. monocytes
5. lymphocytes



Plasma- with anticoagulant
Serum- after coagulation

Plasma (56%)

After centrifugation in Ficoll, leukocytes are found in the "buffy coat" 1%

RBCs

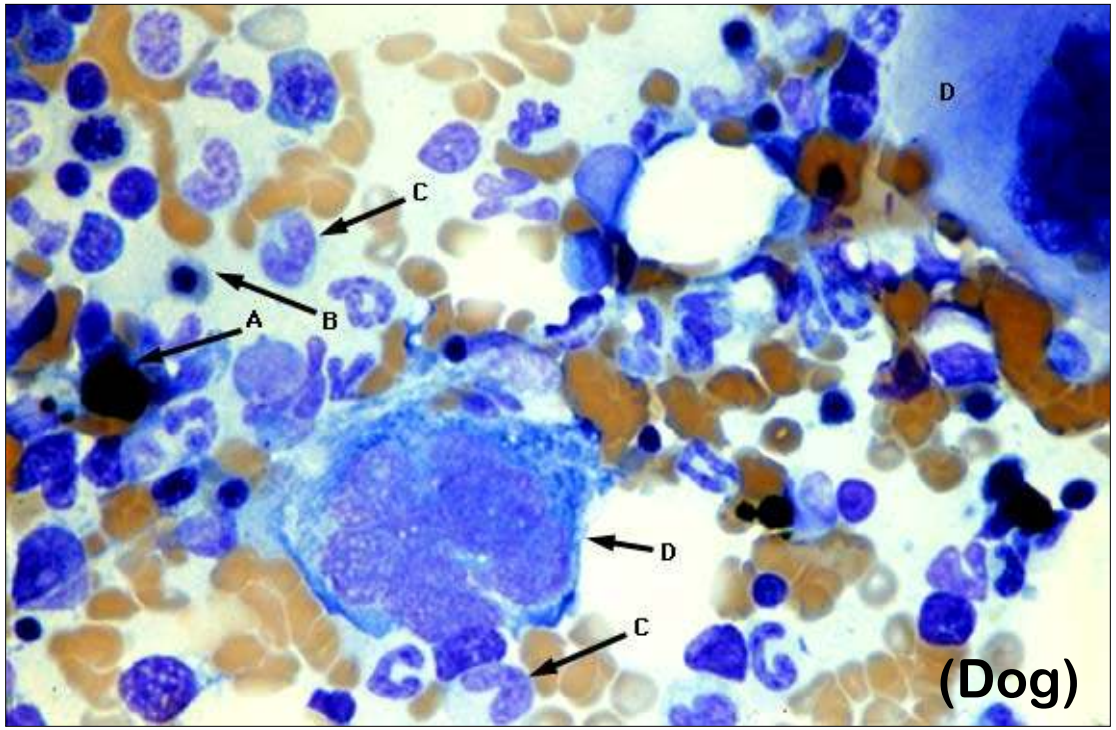
Where do all these cells come from?

The cells of the immune system arise from pluripotent hematopoeitic stem cells (HSC) through two main lines of differentiation

- **Myeloid** lineage produces phagocytes (neutrophils..) and other cells
- **Lymphoid** lineage produces lymphocytes

Hematopoeisis

• Pleuripotent Hematopoeitic Stem Cells give rise to second generation stem cells with restricted lineage potential



A. Hemosiderin: A protein that stores iron in the body, derived chiefly from the hemoglobin released during hemolysis
B. Erythroid precursor
C. Band cells
   - Neutrophil
D. Megakaryocytes
   - platelets

Univ Penn, Vet School, http://cal.nbc.upenn.edu/histo

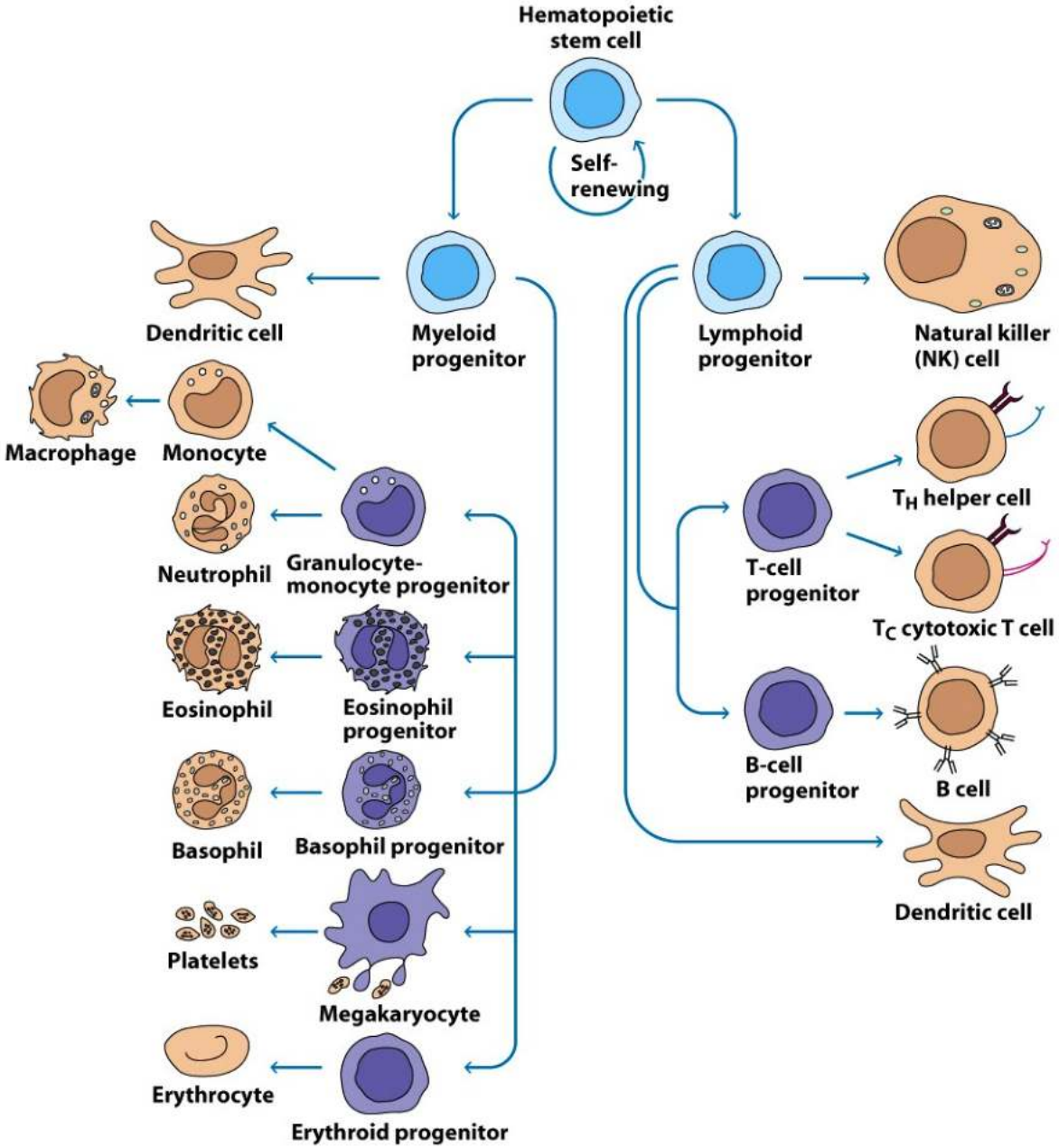
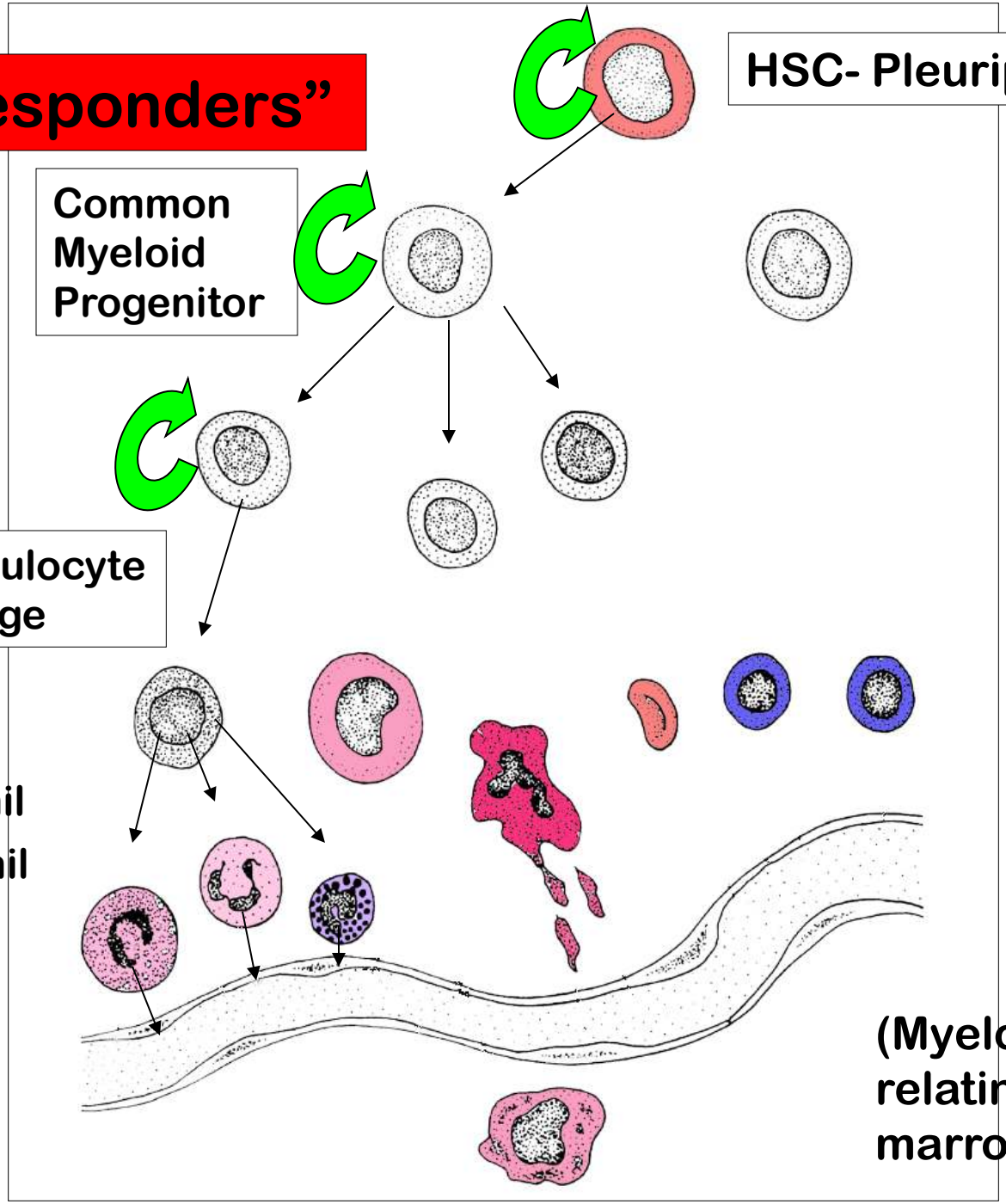


Figure 2-2
Kuby IMMUNOLOGY, Sixth Edition

"First Responders"



(Myeloid = of or relating to the bone marrow)

Granulocytes

- Front line of attack during immune response~ part of innate immune response
- Identified by characteristic staining patterns of “granules”
  - Released in contact with pathogens
  - Proteins with distinct functions: killing, regulation of other cells, tissue remodeling
- All have multilobed nuclei

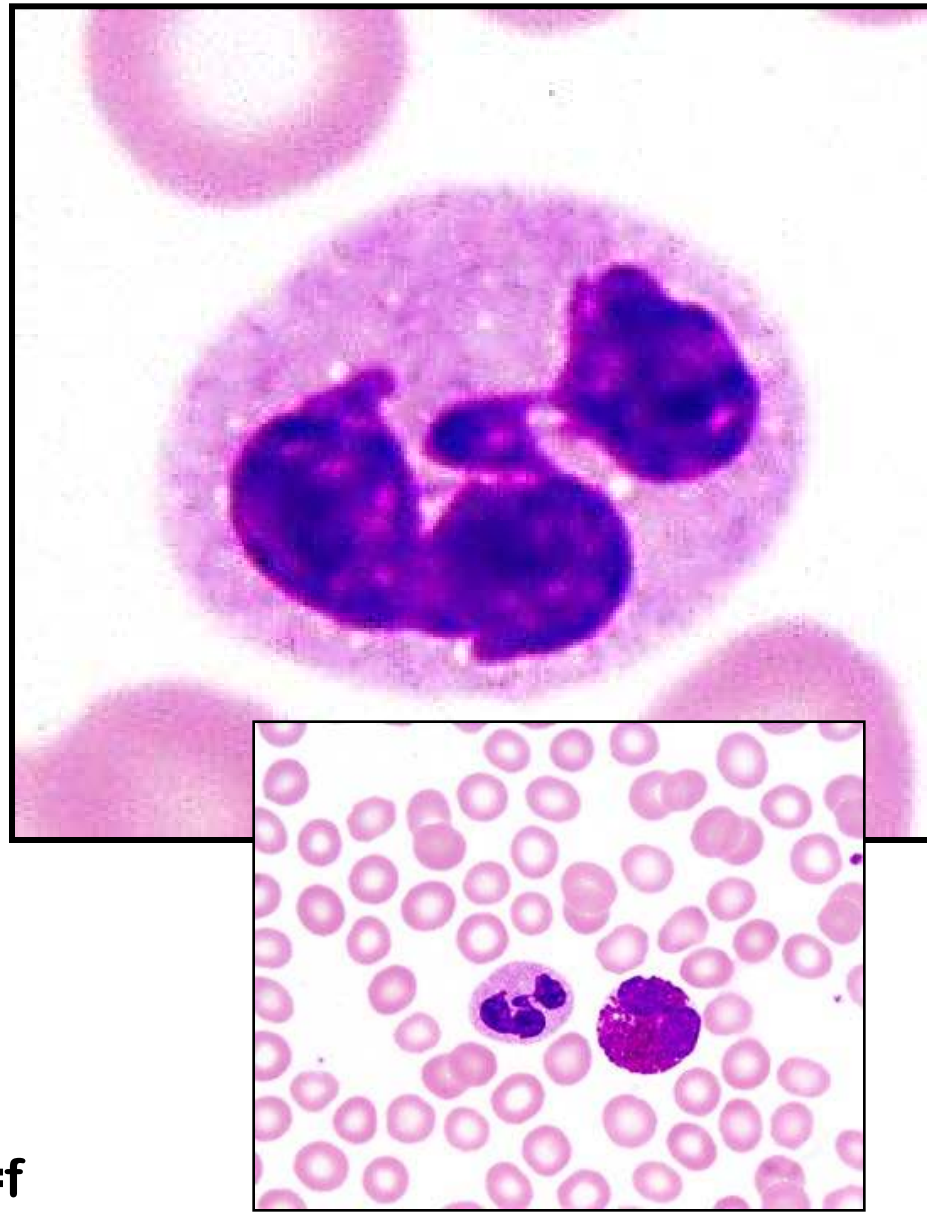
Neutrophils

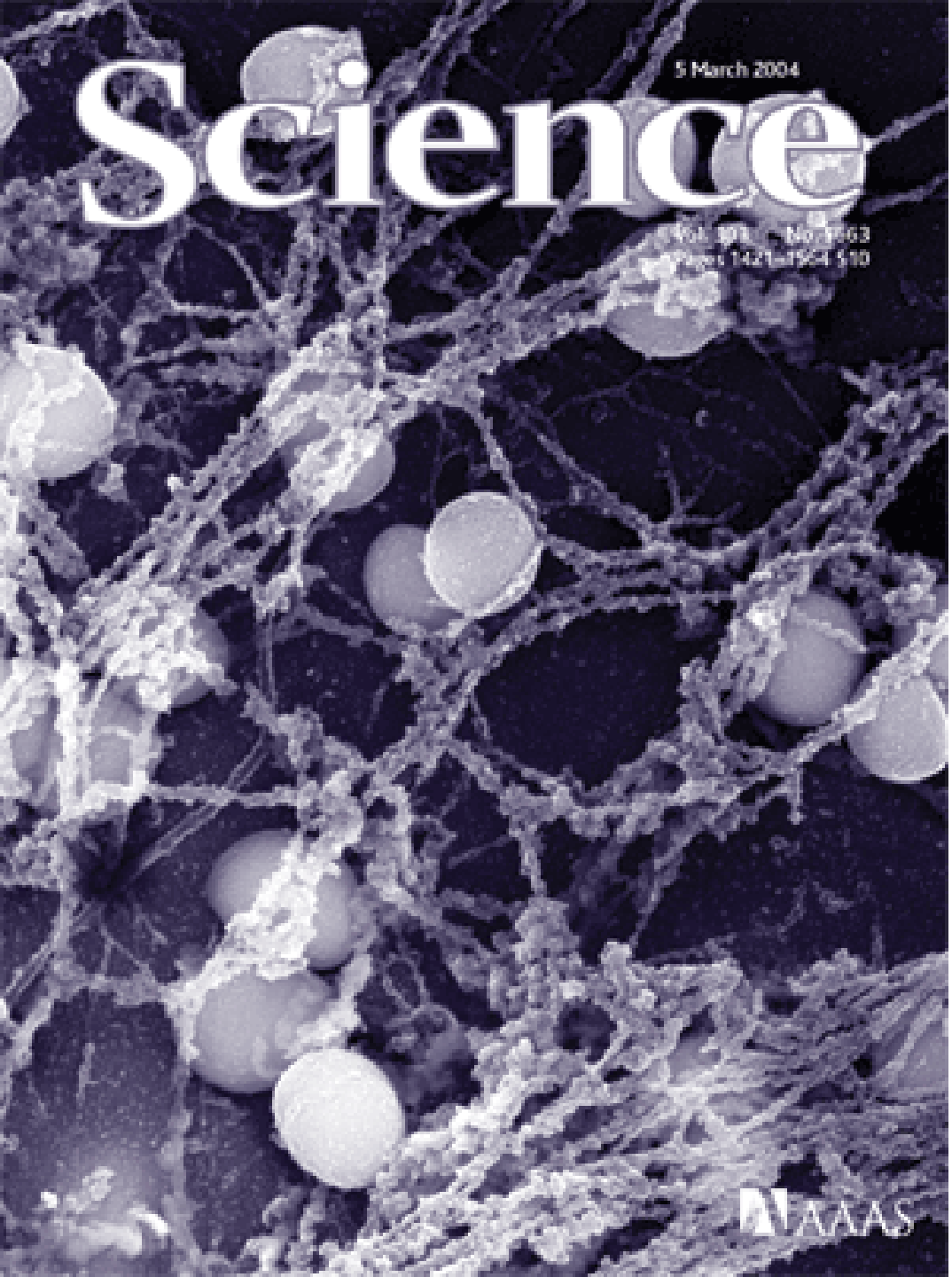
- One of the main effector cells in the innate immune system
- 50-70% of white blood cells
- Released from bone marrow, circulate 7-10 hrs, enter tissues, live only a few days
- Numbers & recruitment increases during infections~ “leukocytosis”~ diagnostic
- shown to kill microorganisms by phagocytosis 100 years ago
- Main cellular component of pus

Neutrophil

- Named based on staining qualities of granules
- Multilobed nucleus= polymorphonuclear leukocyte= PMN
- Neutrophilic granules stain lightly blue to pink
- 7-10 hrs in blood, then migrates into tissues
- First responders- Motile & phagocytic
- "Leukocytosis" indicates infection
- Extracellular "traps"

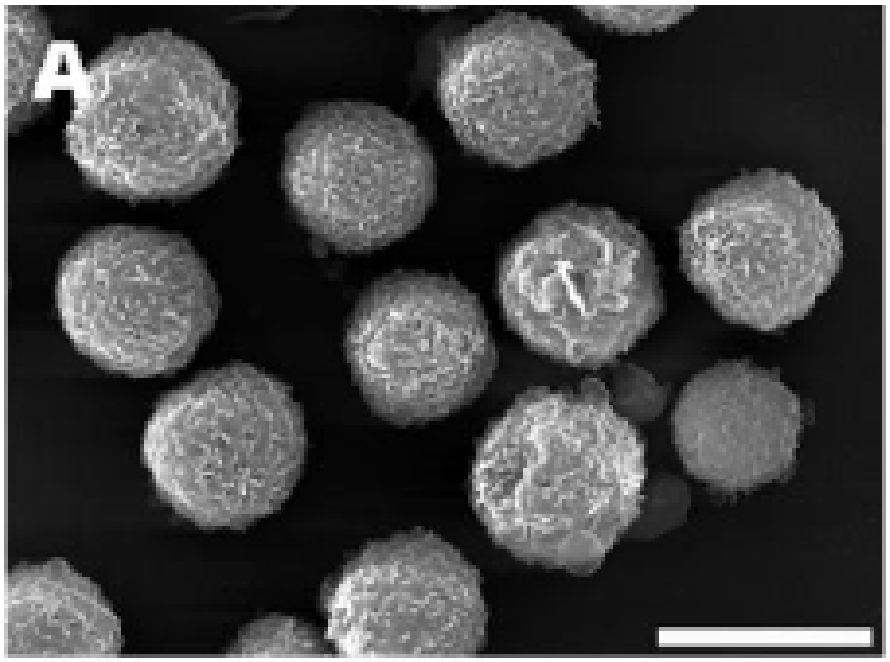
http://www.youtube.com/watch?v=fpOxgAU5fFQ



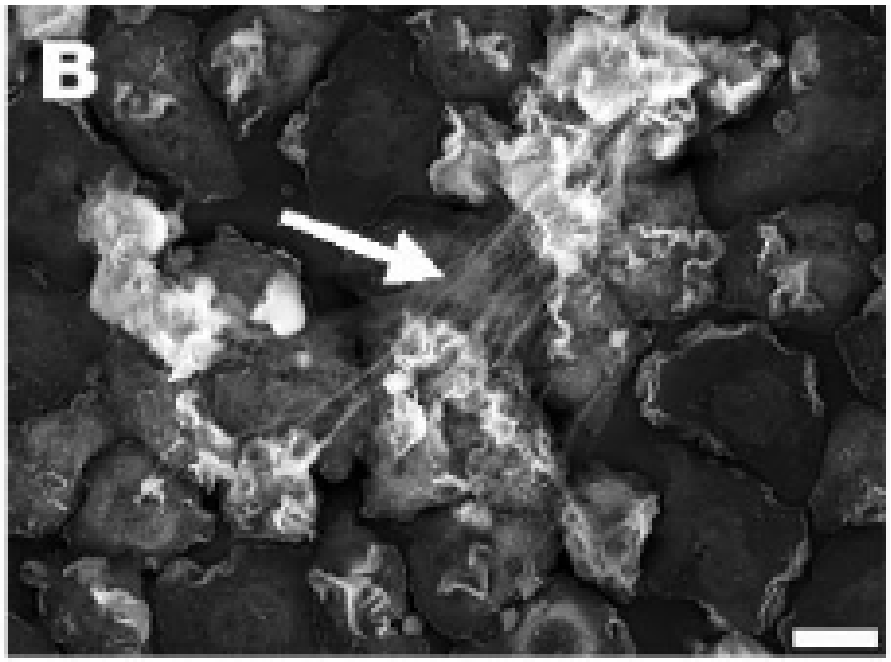


COVER
Scanning electron micrograph of *Staphylococcus aureus* bound to neutrophil extracellular traps (NETs). These novel structures formed by activated neutrophils can disarm and kill bacteria before they reach host cells

NETS



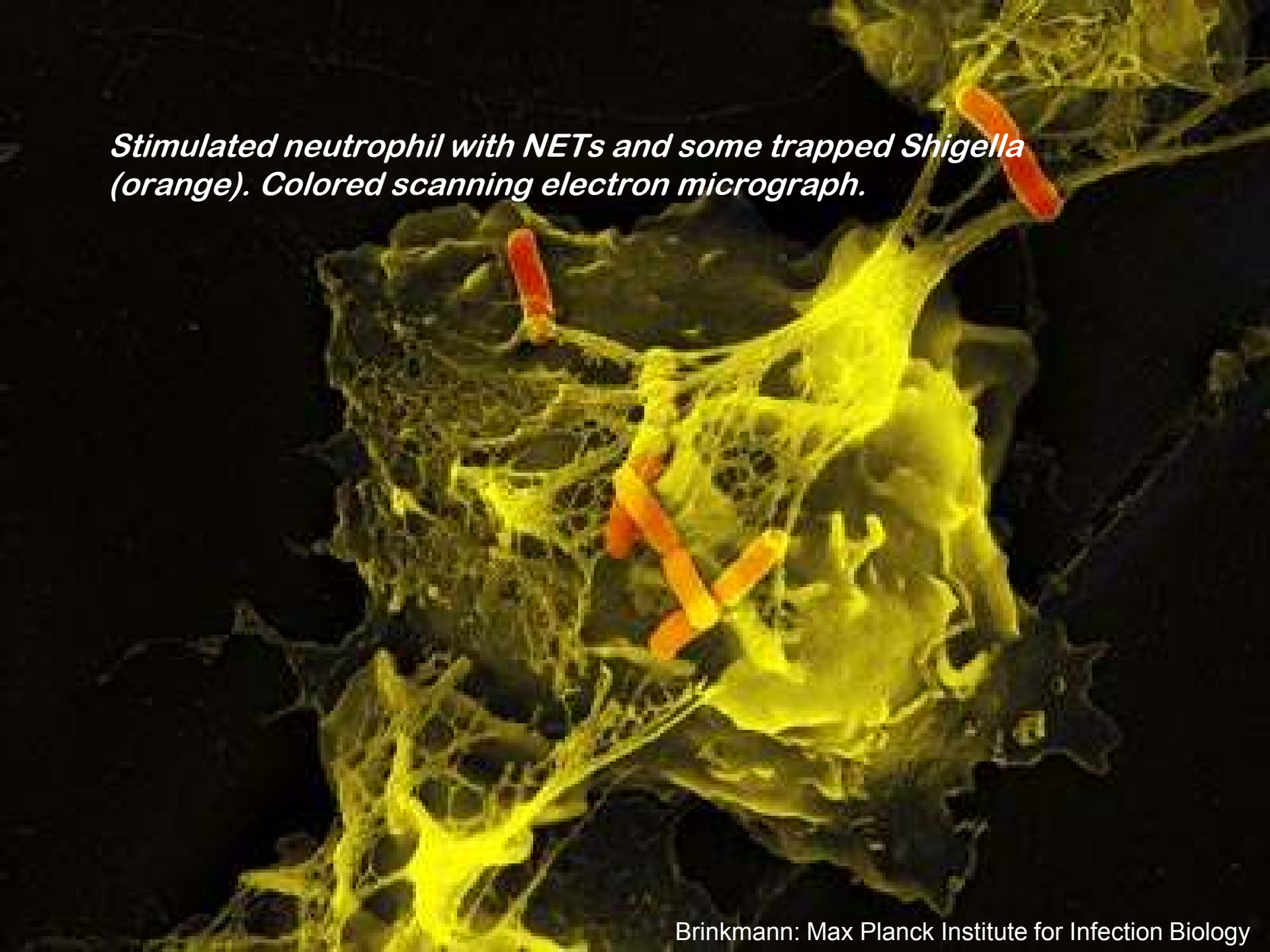
neutrophils resting



neutrophils activated

Brinkman/Zychlinsky Nat Rev Micro 5: 2007
“Beneficial suicide: why neutrophils die to make NETS”

Brinkmann et al, Science 303, 2004

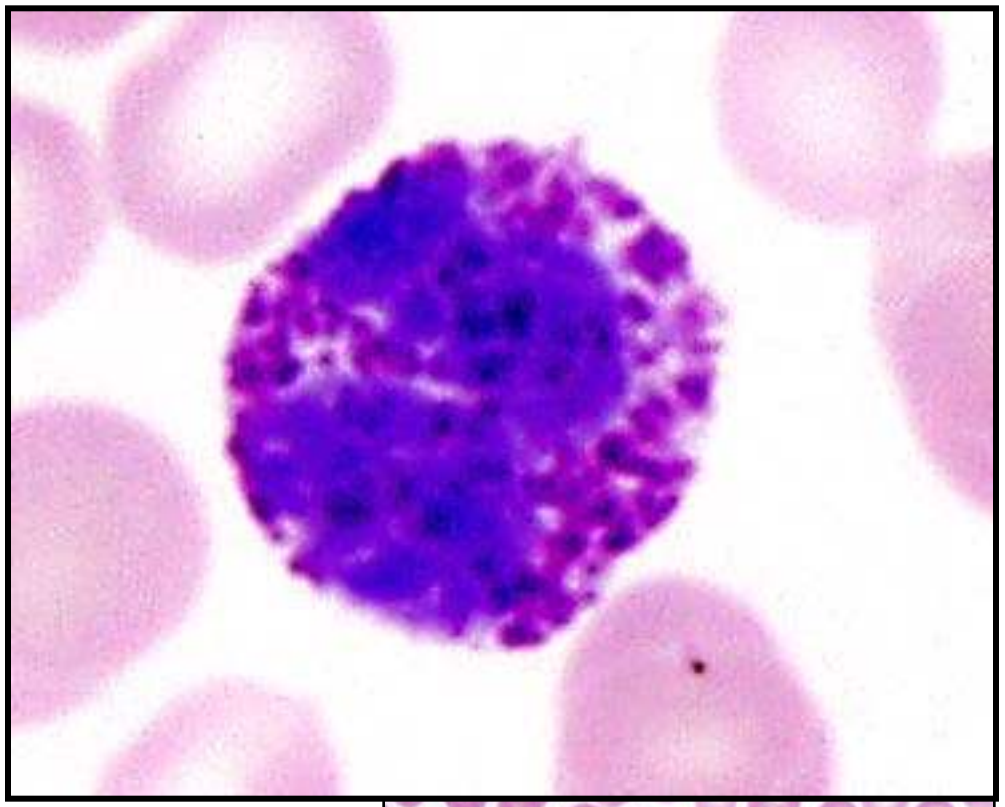


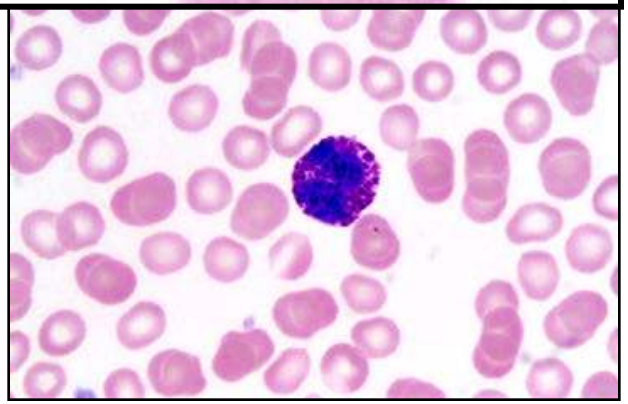
Stimulated neutrophil with NETs and some trapped Shigella (orange). Colored scanning electron micrograph.

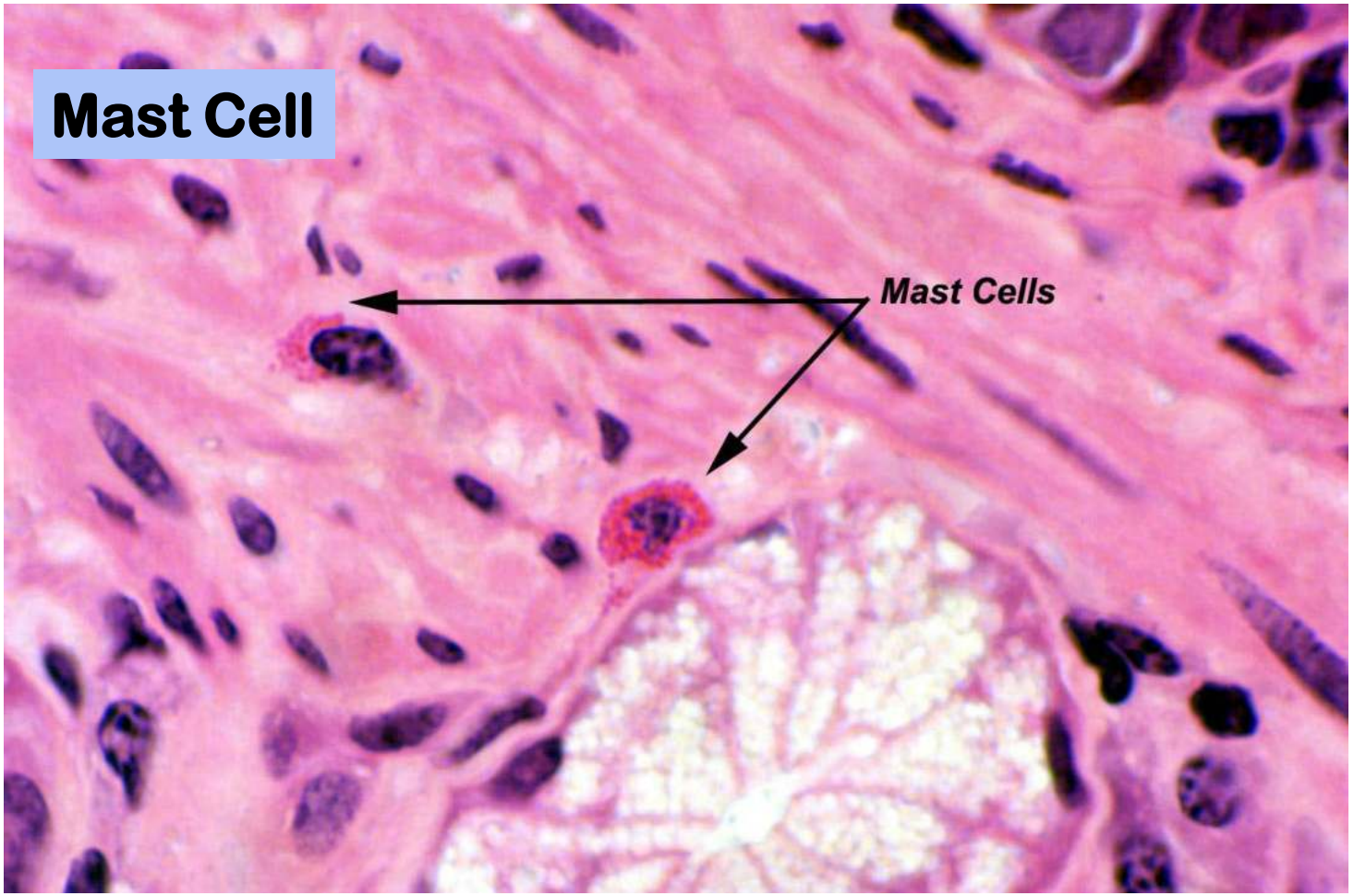
Brinkmann: Max Planck Institute for Infection Biology

Basophil

- <1% all leukocytes
- Non-phagocytic
- Nucleus obscured by coarse blue (H&E) granules
- Important in some allergic responses
- Critical to response to parasites
- Bind circulating Abs and release histamine-increasing permeability of blood vessels



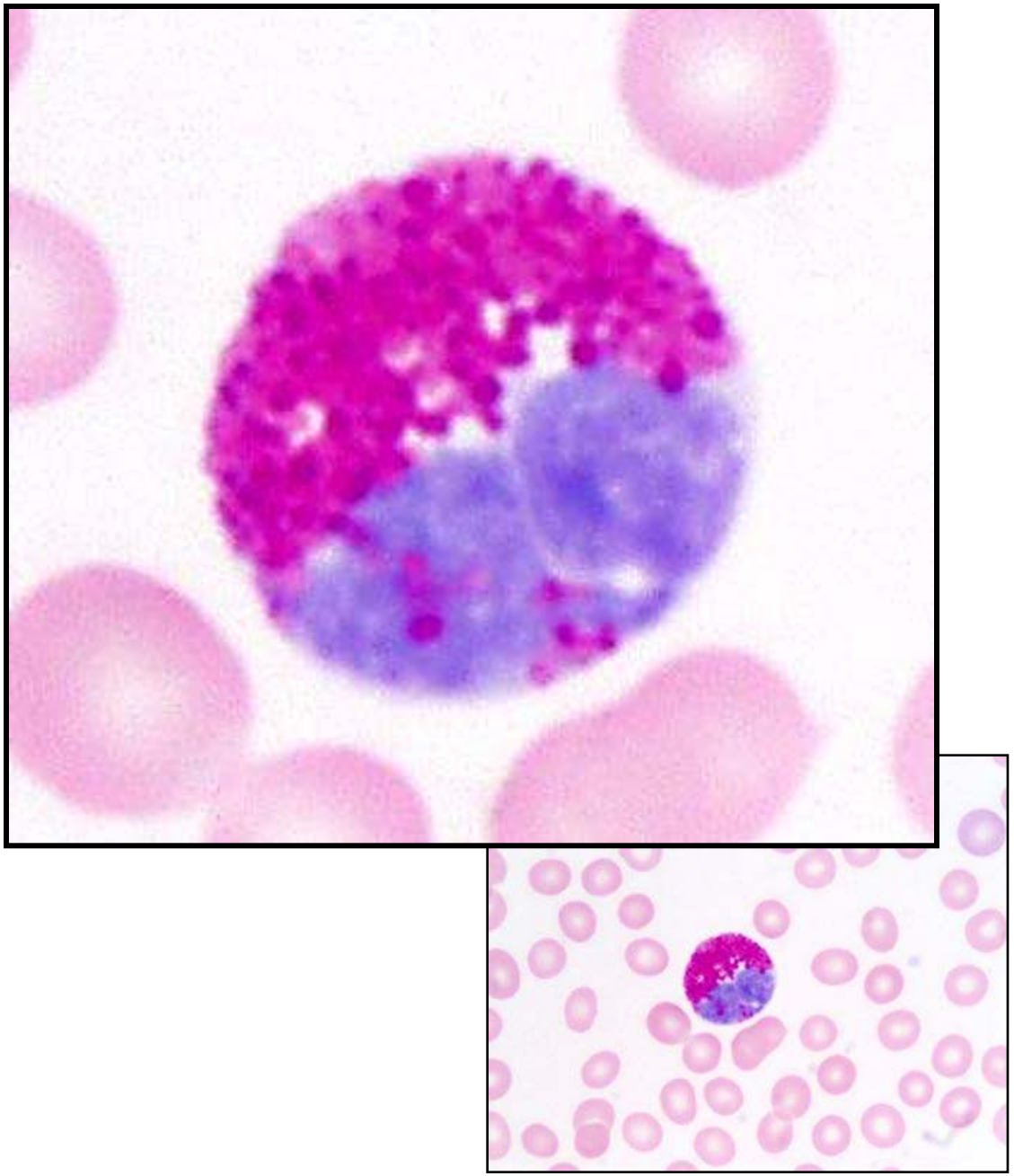




- Leave bone marrow as undifferentiated cells and mature in tissues; histamine
- May be related to basophils (?)

Eosinophil

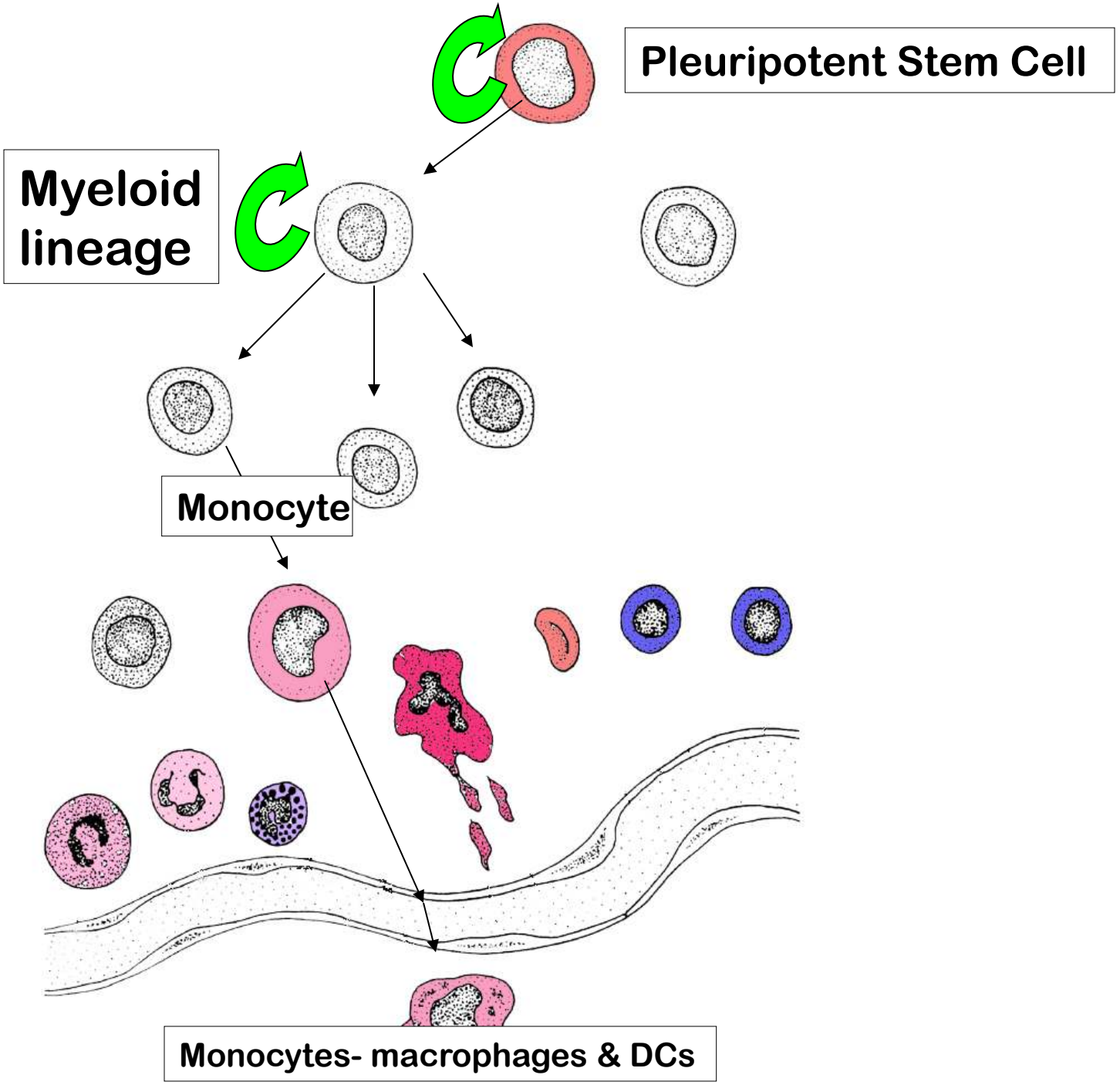
- Bilobed nuclei
- Motile, phagocytic
- Killing of antibody coated parasites
- Degranulation of substances that kill parasites, worms



Myeloid antigen presenting cells:

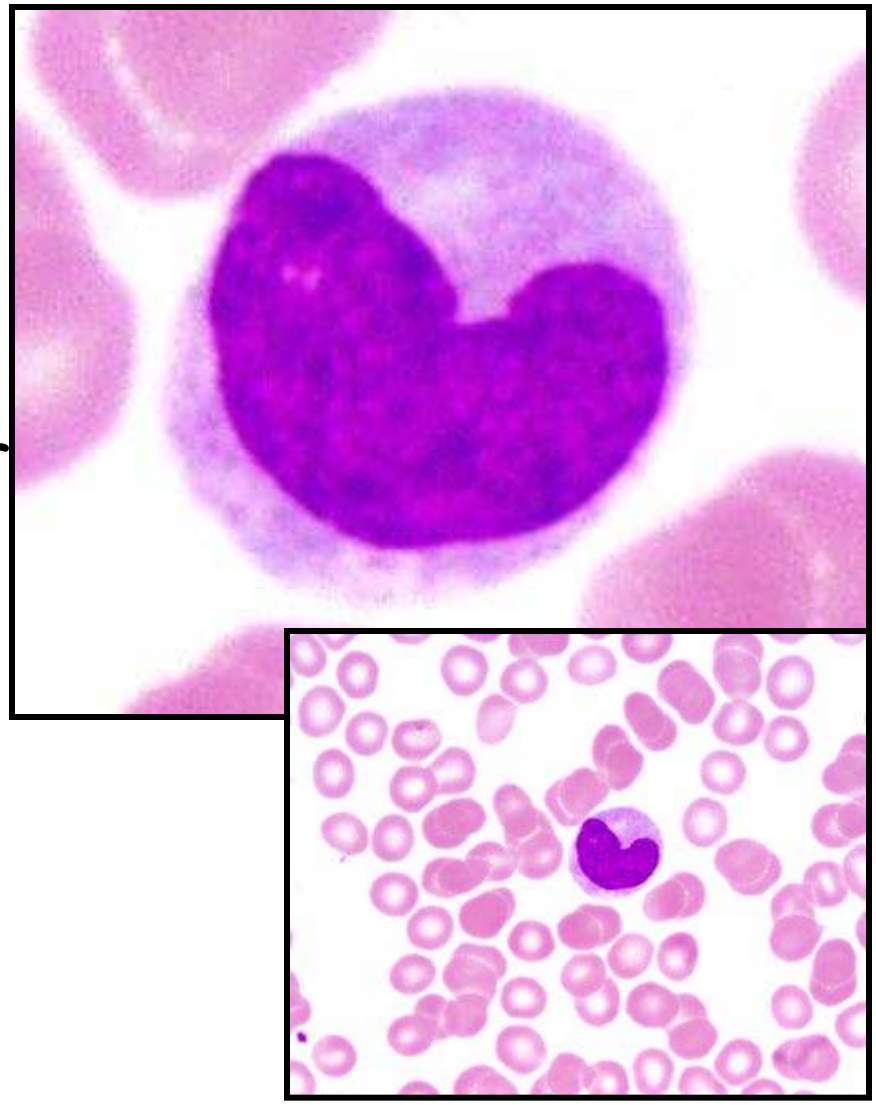
Monocytes, macrophages, dendritic cells

- Phagocytic
- Ingest, digest into peptides, present on cell surface
- Bridge between innate and adaptive immune responses
- Make contact with antigens in periphery and then interact with lymphocytes in lymph node
- Secrete proteins that attract and activate other immune cells


Pleuripotent Stem Cell
Myeloid lineage
Monocyte
Monocytes- macrophages & DCs

Monocyte

- Mononuclear
- Circulate in blood~ 8 hrs
- Bean-shaped nucleus
- Enter tissues and become fully mature macrophages or dendritic cells
  - Enlarges
  - Becomes phagocytic
- Free vs fixed tissue mΦ
  - Special names in different organs- Kupffer cells-liver
- Digest and/or *present* Ag
- Surface receptors for Abs (opsinized Ags)



Macrophage

- Monocytes enter tissues and become fully mature macrophages or dendritic cells
  - Enlarge
  - Become phagocytic
- Free vs fixed tissue mΦ
  - Special names in different organs- Kupffer cells-liver
- Digest and/or *present* Ag
- Surface receptors for Abs (opsinized Ags)

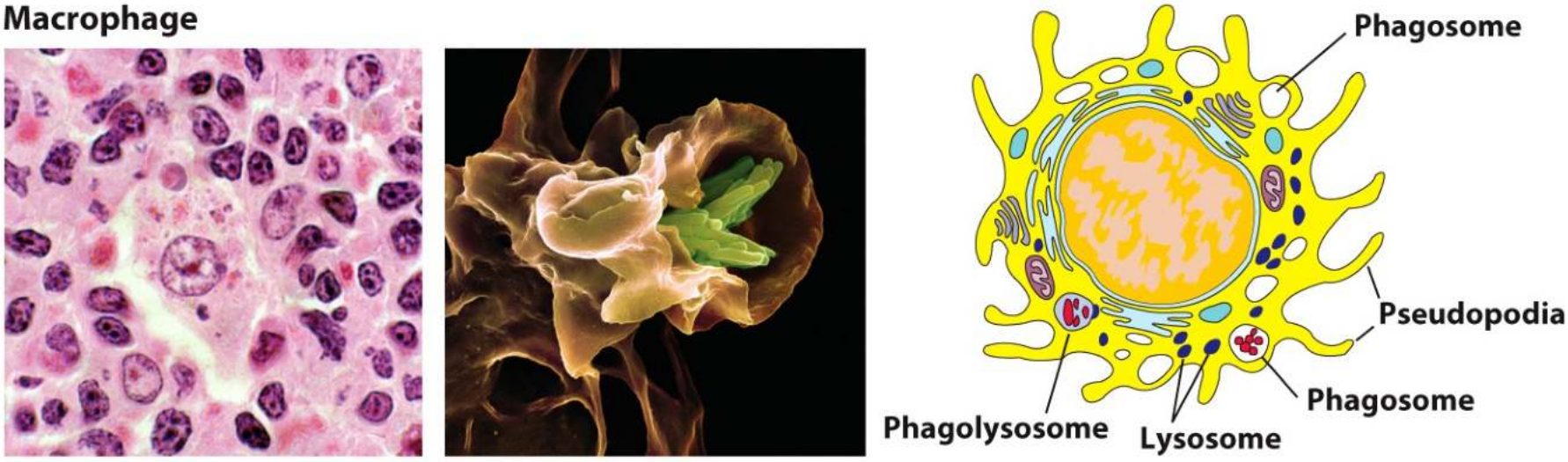


Figure 2-3b
Kuby Immunology, Seventh Edition

Dendritic cells:

heterogeneous myeloid & lymphoid origins

- Best APC for presenting to naïve T-cells
- Ralph Steinman discovered them in mid 1970's; just received Nobel Prize 2011
- Critical
- Named for long processes; actively extend and retract sampling Ags & examining T cells
- Capture Ag in one place- then migrate- present Ag in another place (eg. LN)
- Immature to mature; change in functionality

Dendritic cell

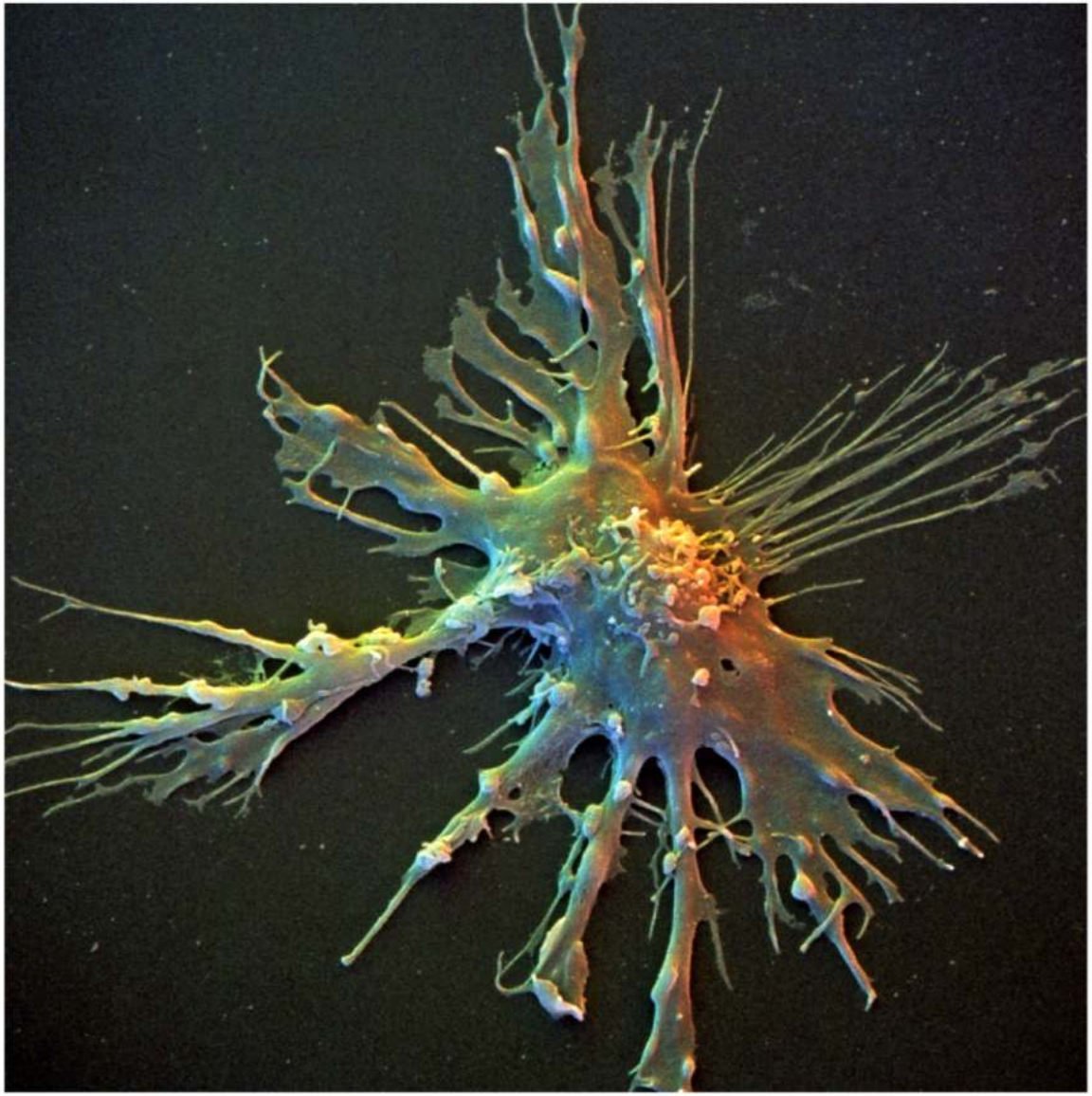
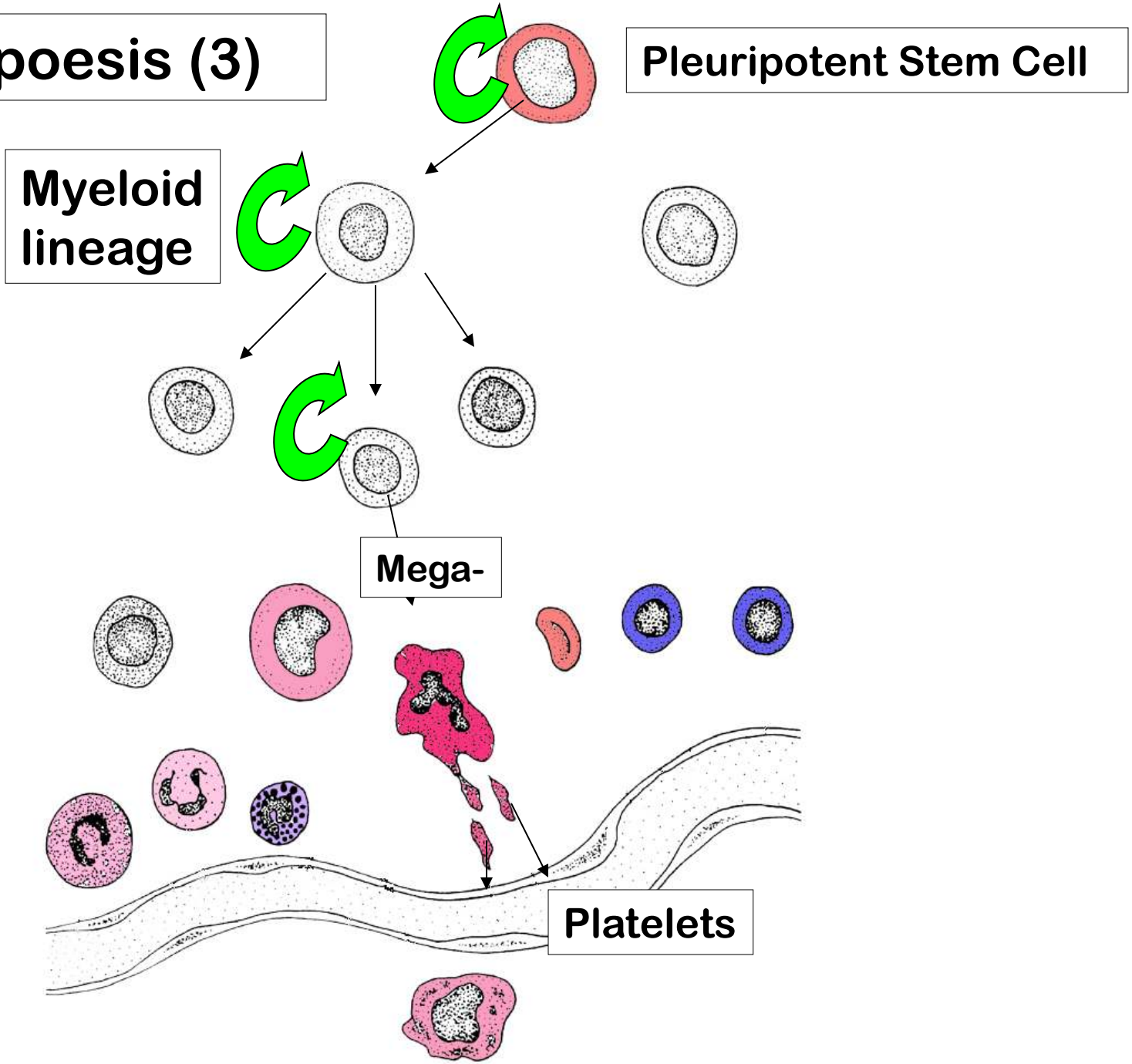
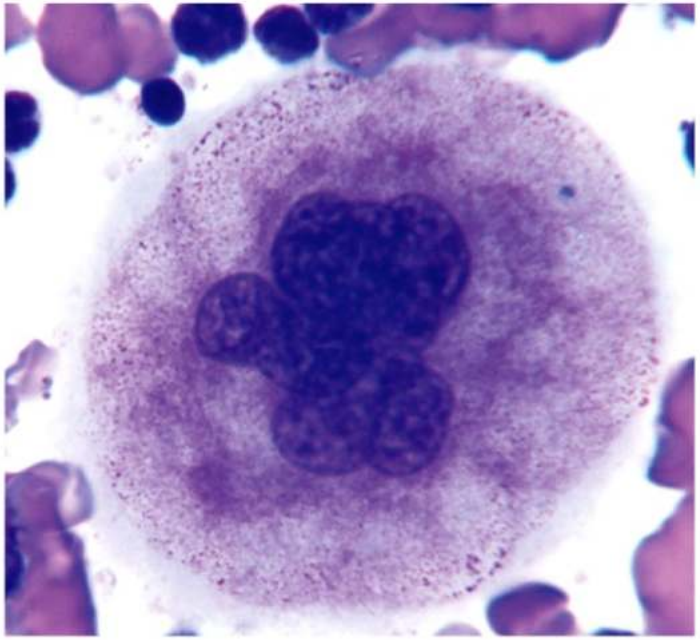


Figure 2-3c part 1
Kuby Immunology, Seventh Edition

Hematopoesis (3)



Megakaryocyte



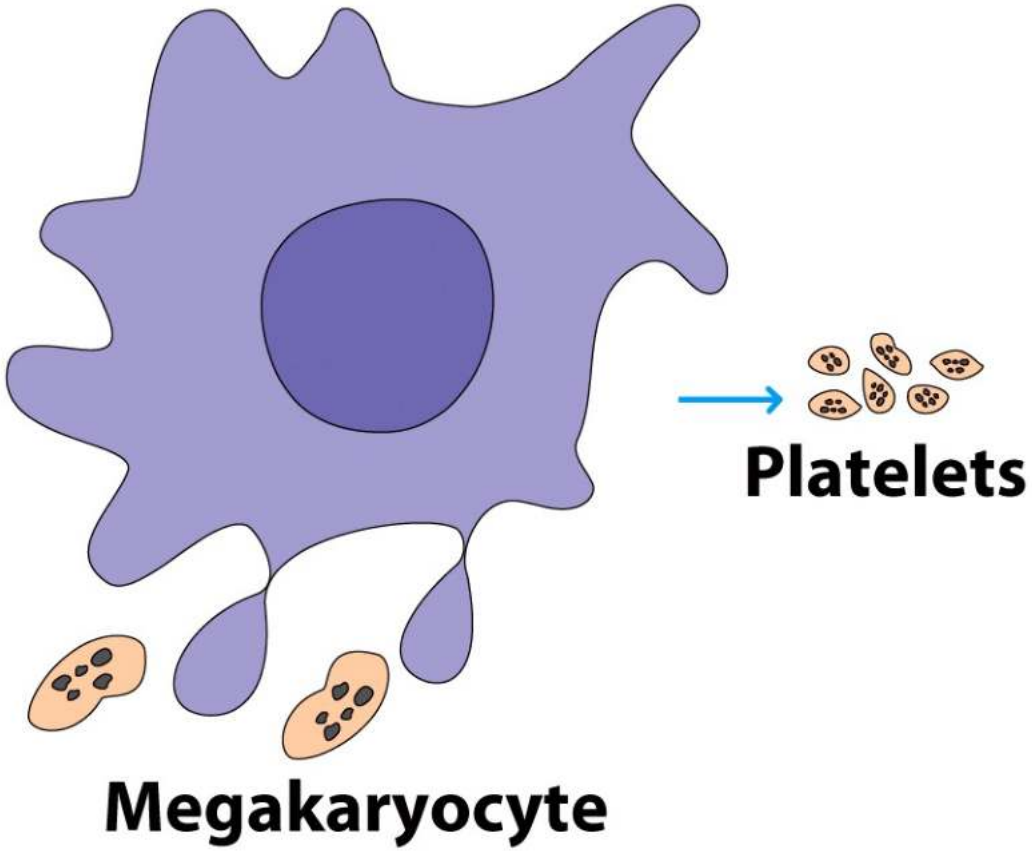
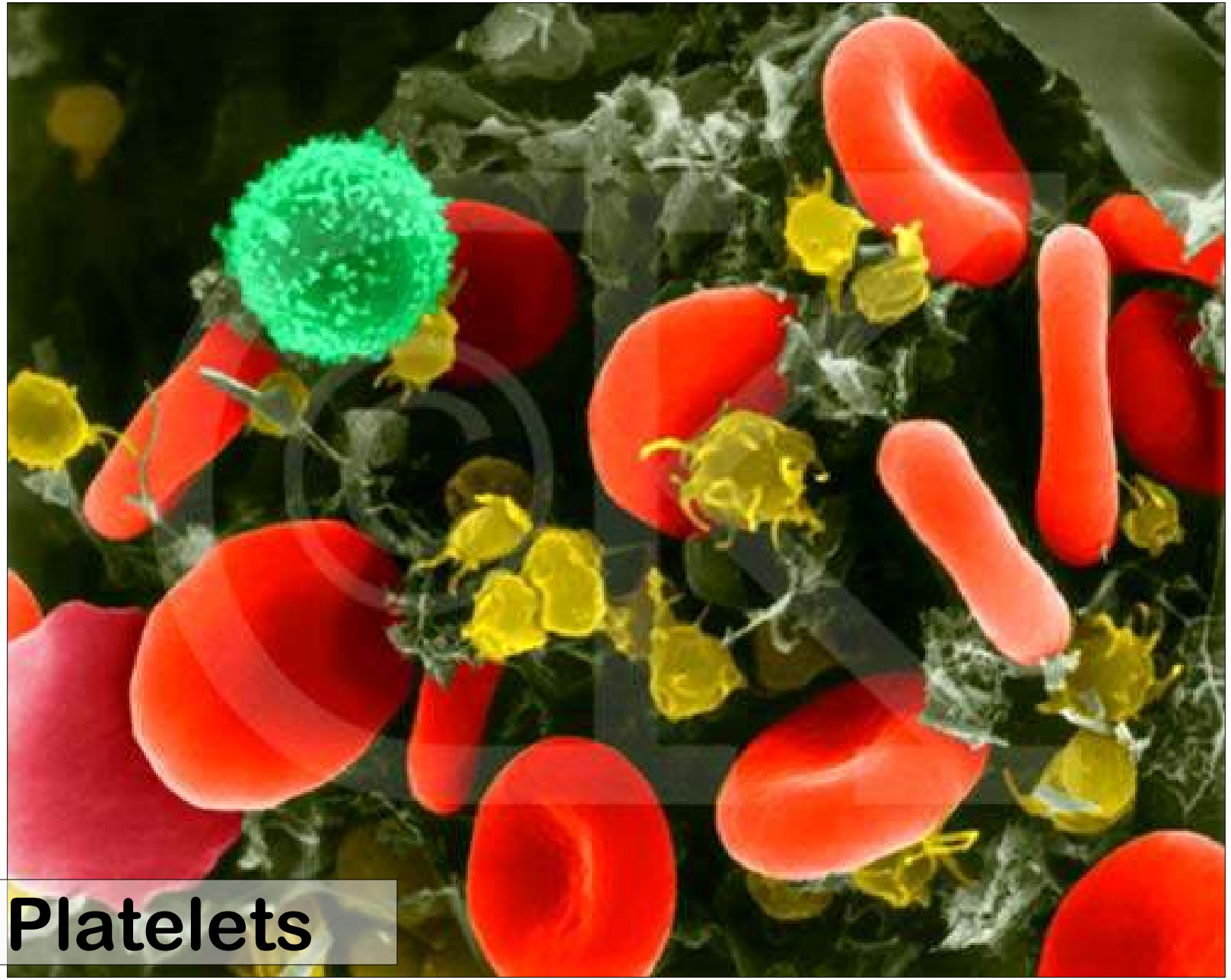
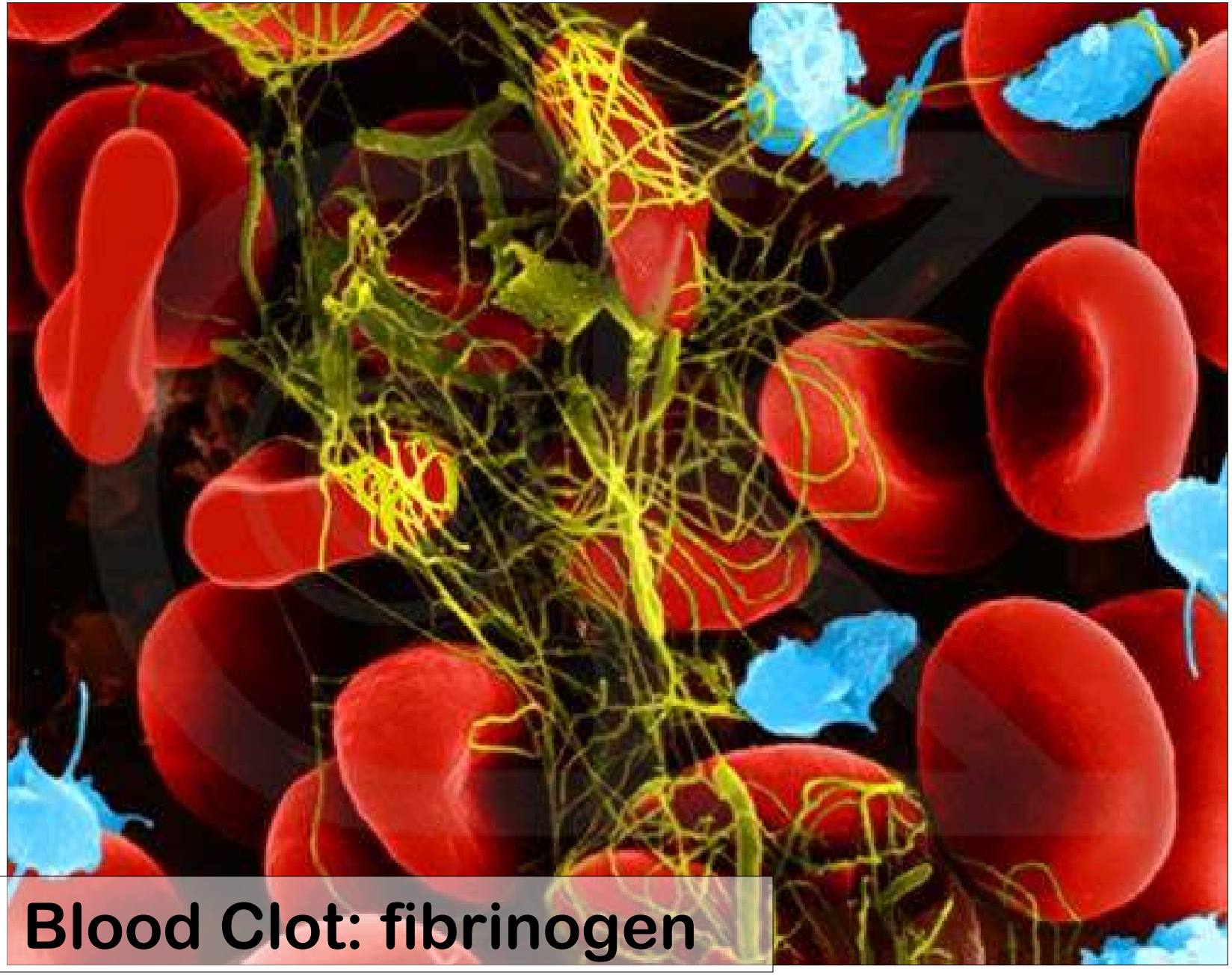


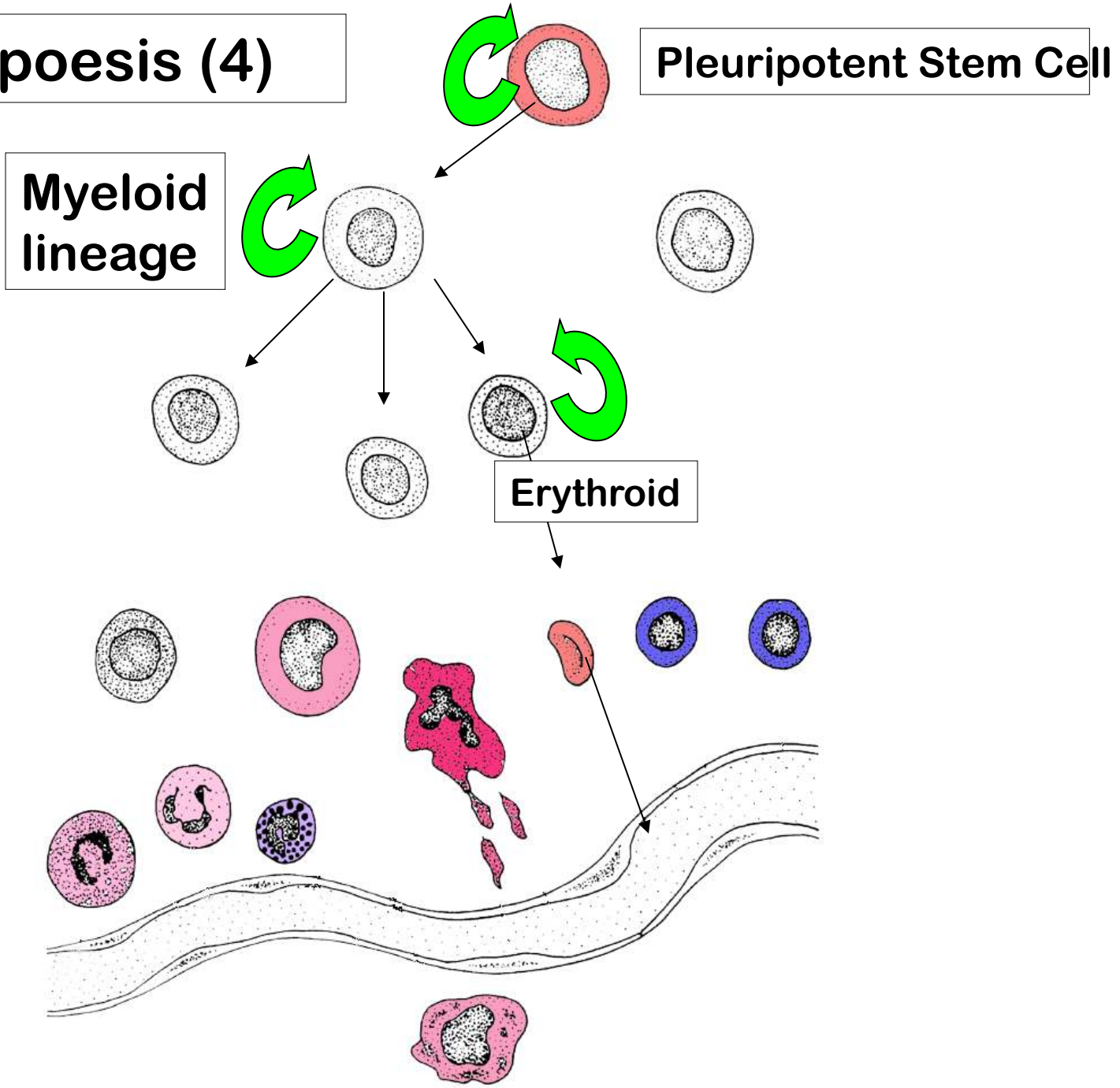
Figure 2-3d
Kuby Immunology, Seventh Edition


Platelets

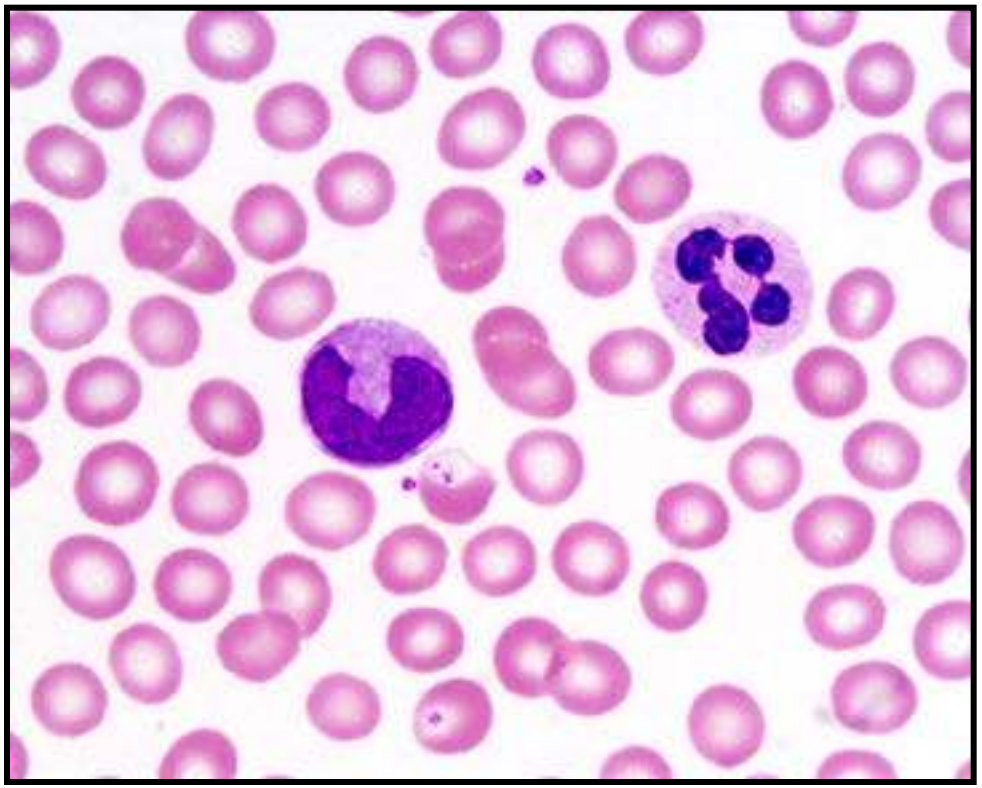


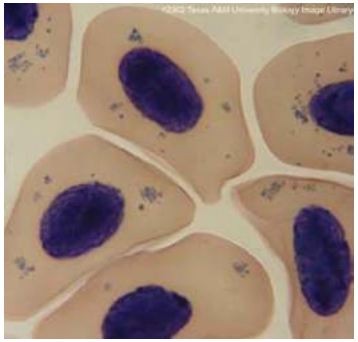
Blood Clot: fibrinogen

Hematopoesis (4)



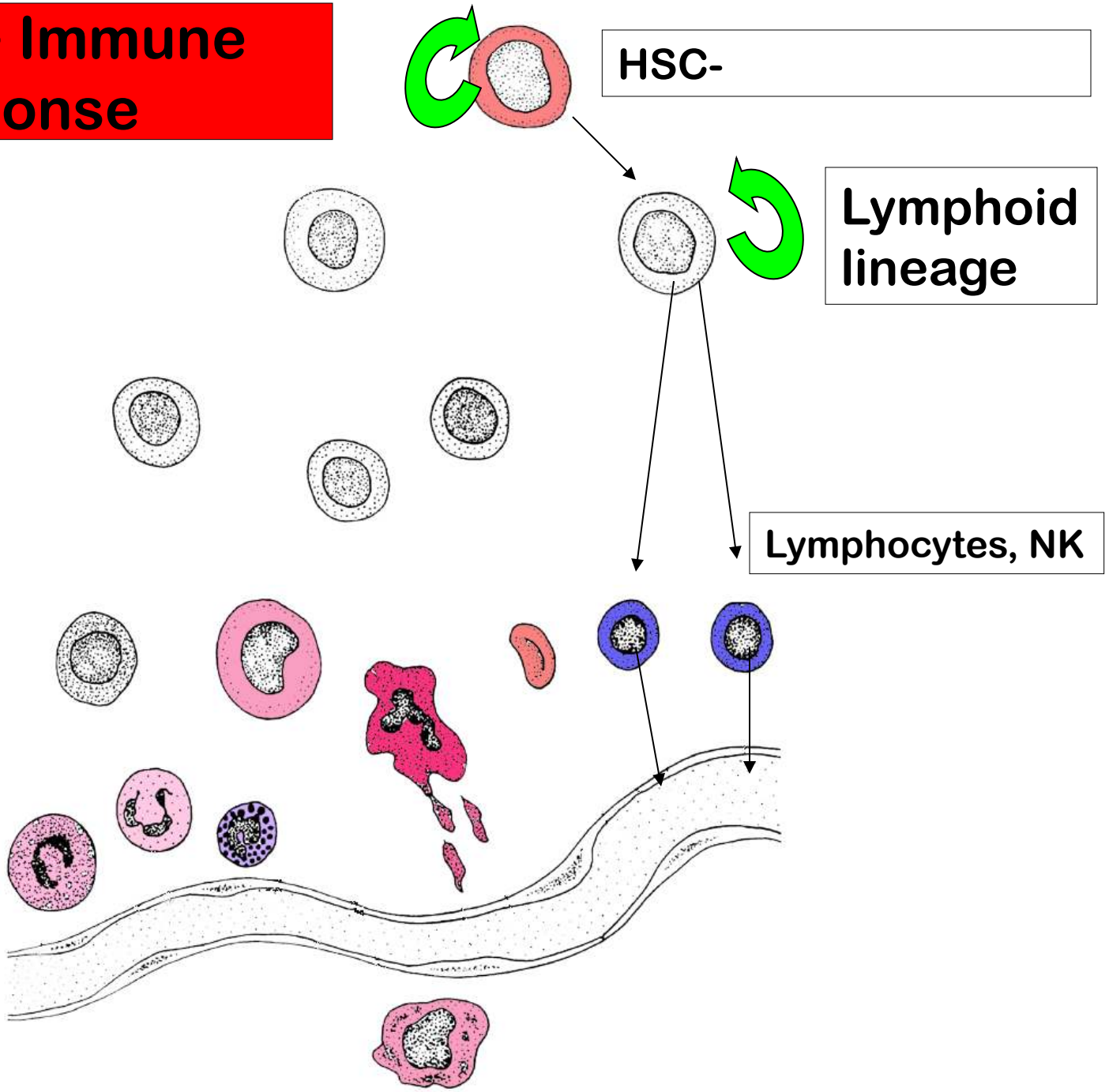
Mature human and mouse RBCs have no nuclei





Salamander RBCs

Adaptive Immune Response

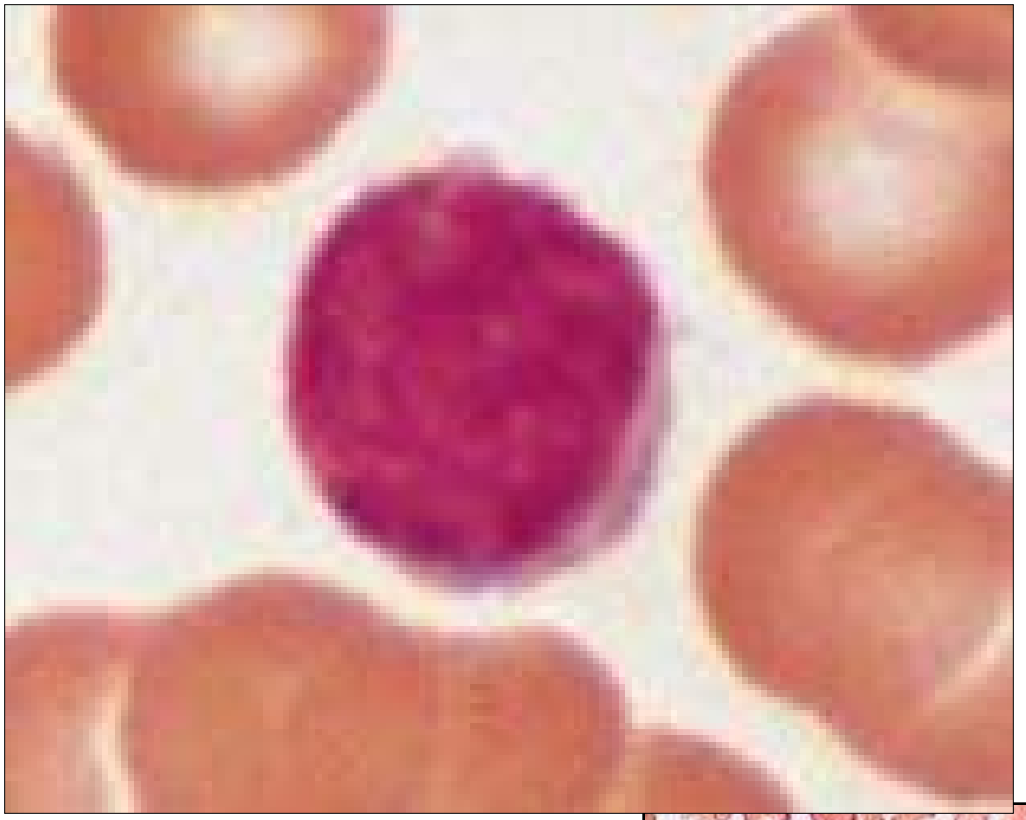


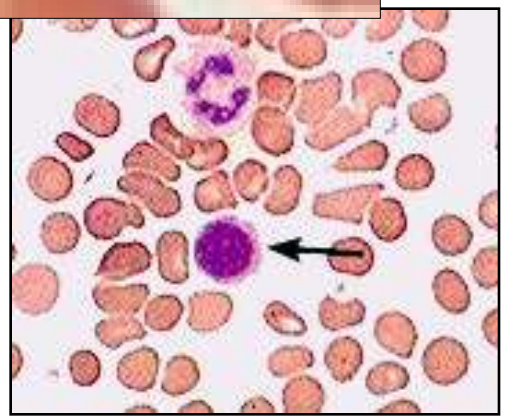
Lymphocytes: 3 types

- 20-40% of WBC
- Cannot be distinguished morphologically
- T-cells
  - helper CD4+ recognize Ag in context of MHCII
  - cytotoxic CD8+ recognize Ab in MHCI
- B-cells
  - become antibody producing plasma cells
- NK cells
  - part of the innate immune response

T and B Lymphocytes

- Large nucleus with dense heterochromatin
- Thin rim of cytoplasm
- Recognizes specific antigenic determinants
- Therefore are responsible for specificity and memory of the adaptive immune response





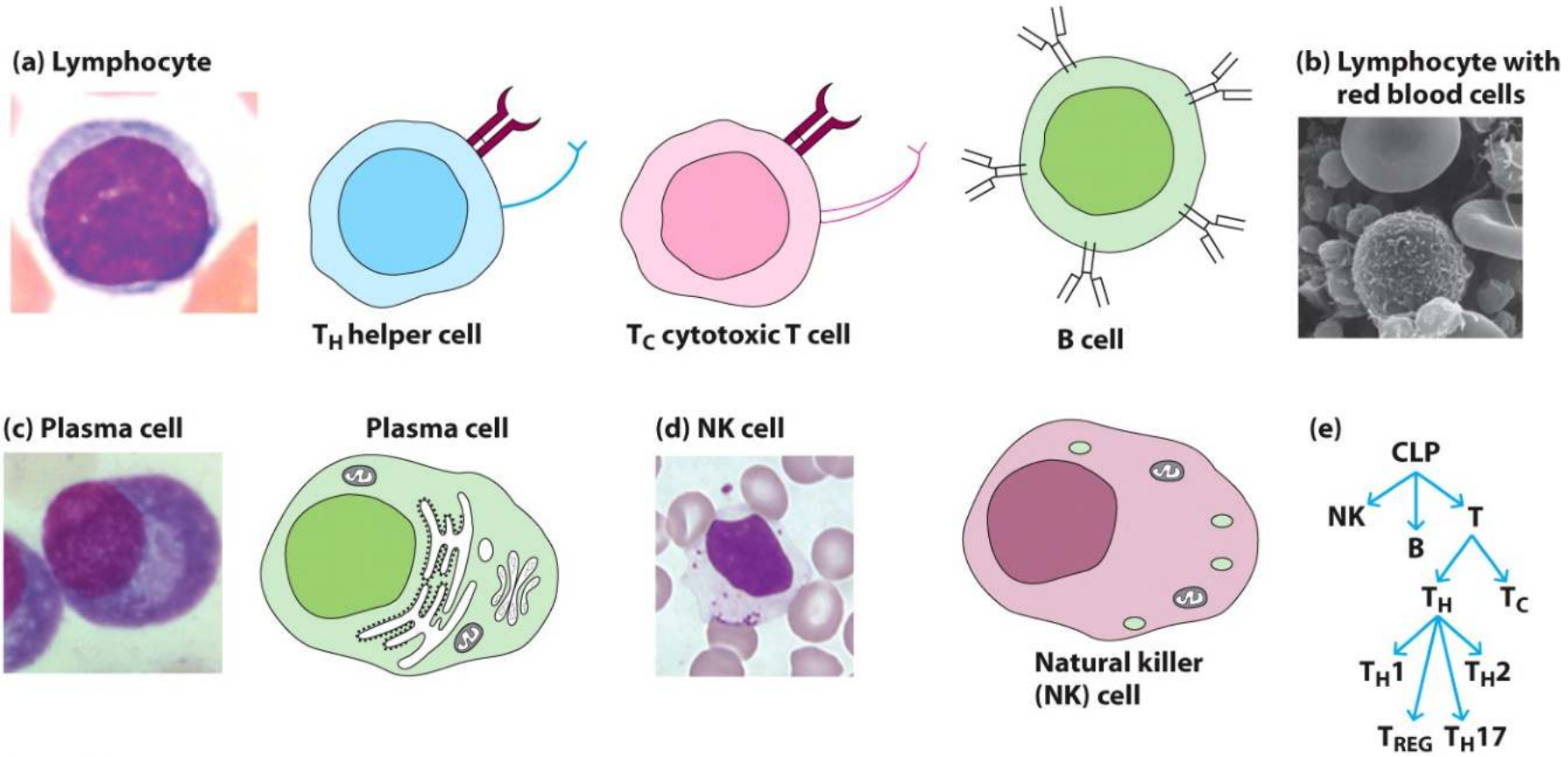


Figure 2-4
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TABLE 2-5 Common CD markers used to distinguish functional lymphocyte subpopulations

CD designation*	Function	B cell	T cell		NK cell
			T_H	T_C	
CD2	Adhesion molecule; signal transduction	−	+	+	+
CD3	Signal transduction element of T-cell receptor	−	+	+	−
CD4	Adhesion molecule that binds to class II MHC molecules; signal transduction	−	+ (usually)	− (usually)	−
CD5	Unknown (subset)	−	−	+	+
CD8	Adhesion molecule that binds to class I MHC molecules; signal transduction	−	− (usually)	+ (usually)	+ (variable)
CD16 (FcγRIII)	Low-affinity receptor for Fc region of IgG	−	−	−	+
CD21 (CR2)	Receptor for complement (C3d) and Epstein-Barr virus	+	−	−	−
CD28	Receptor for costimulatory B7 molecule on antigen-presenting cells	−	+	+	−
CD32 (FcγRII)	Receptor for Fc region of IgG	+	−	−	−
CD35 (CR1)	Receptor for complement (C3b)	+	−	−	−
CD40	Signal transduction	+	−	−	−
CD45	Signal transduction	+	+	+	+
CD56	Adhesion molecule	−	−	−	+

*Synonyms are shown in parentheses.

Table 2-5
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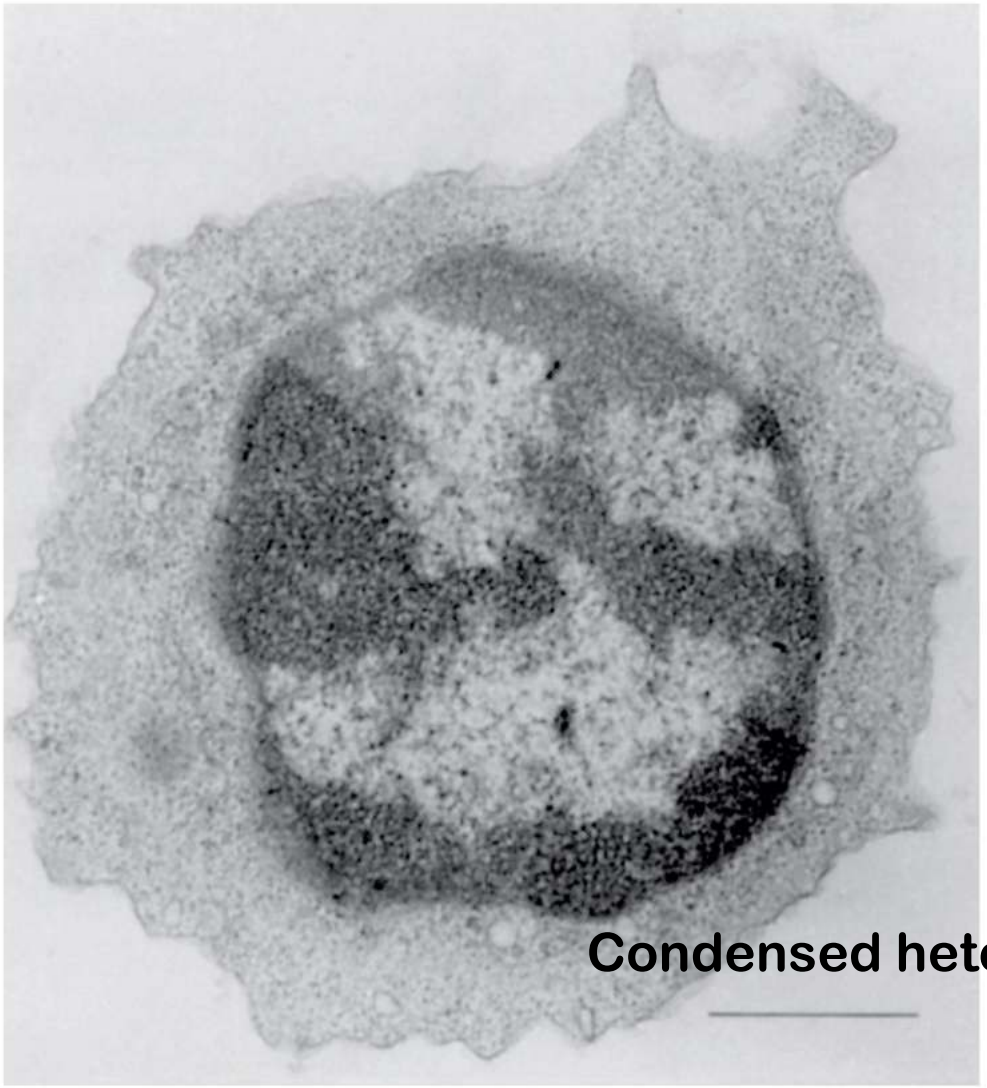
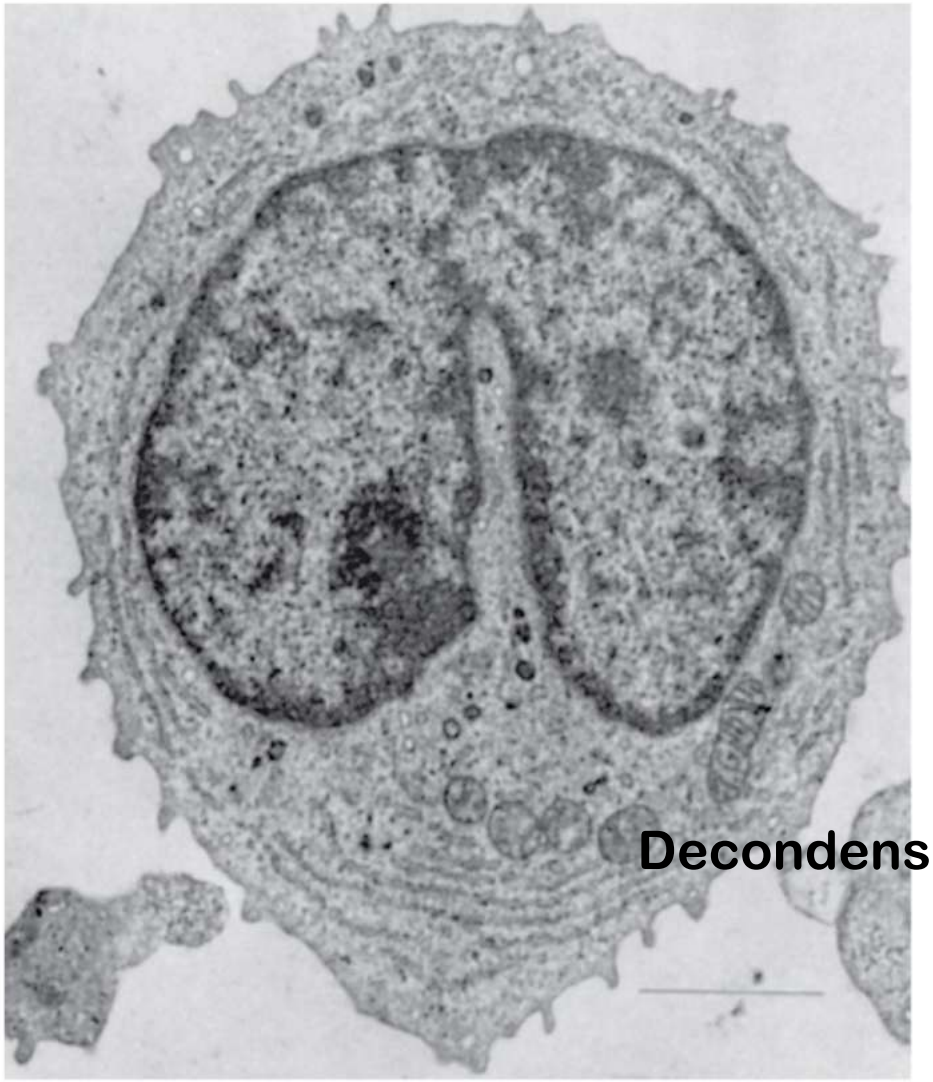


Condensed heterochromatin= resting

Small lymphocyte (T or B)
6 μm diameter

Figure 2-6b part 1
Kuby IMMUNOLOGY, Sixth Edition



Decondensed chromatin= active

Blast cell (T or B)
15 μm diameter

Figure 2-6b part 2
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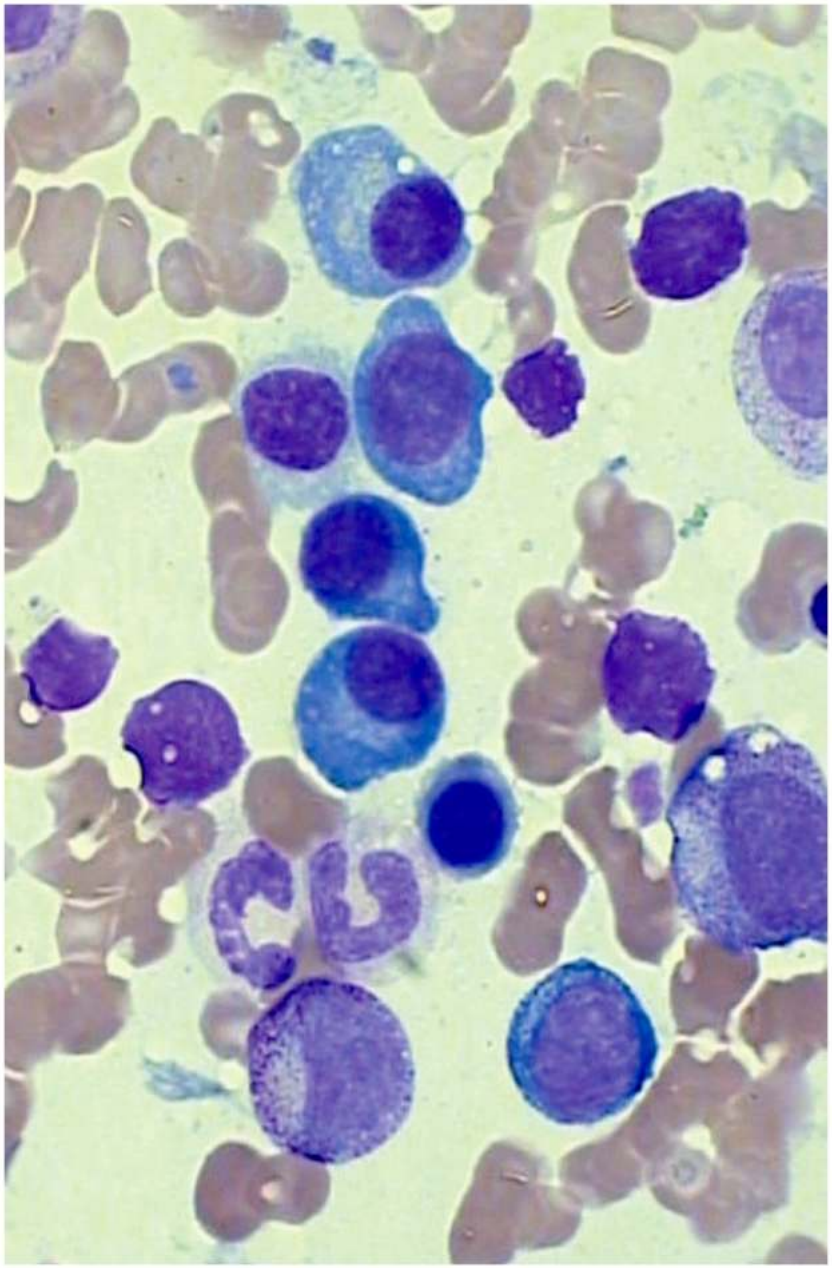
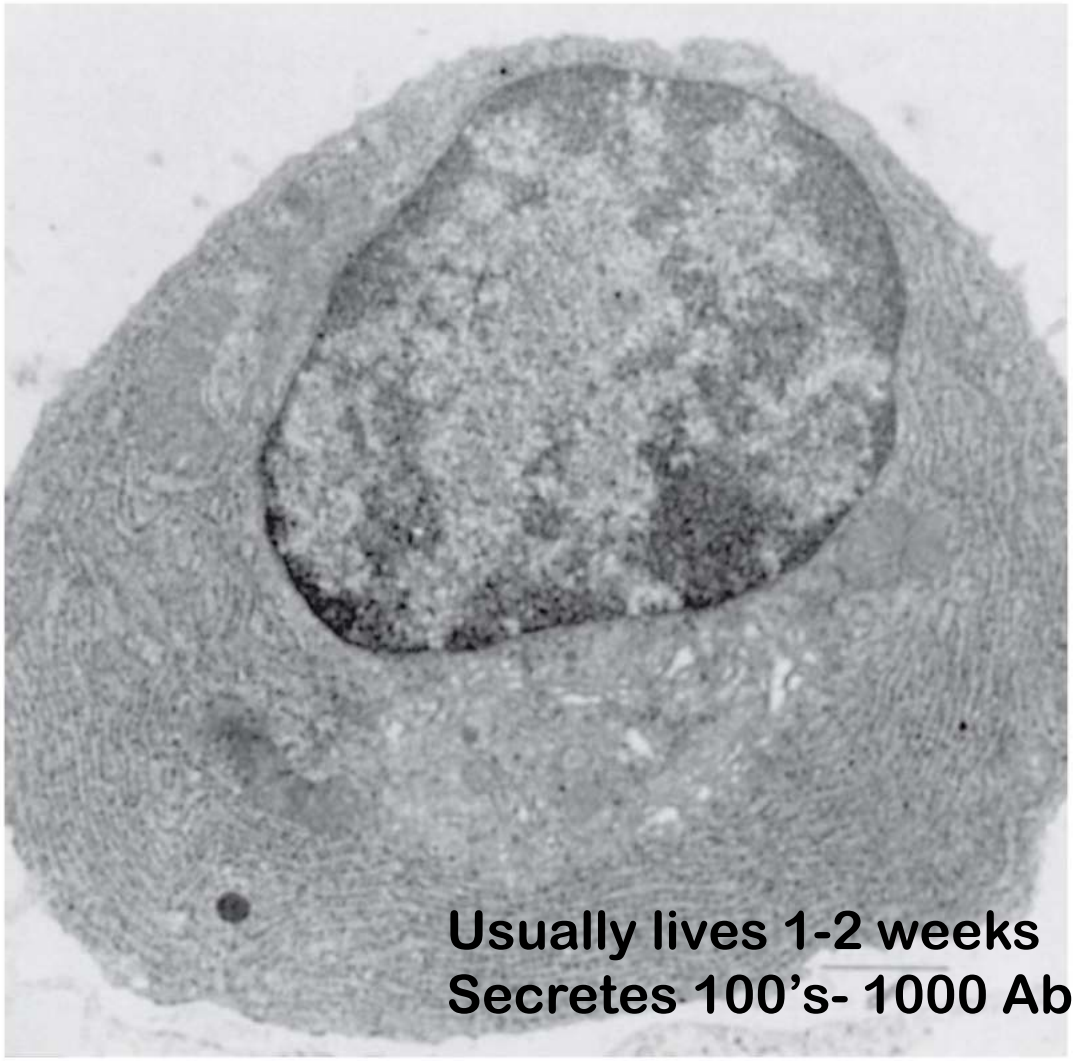


Plasma cell
Perinuclear golgi and abundant layers of endoplasmic reticulum

Figure 2.8e *The Biology of Cancer* (© Garland Science 2007)



Usually lives 1-2 weeks
Secretes 100's- 1000 Ab molecules/sec

Plasma cell (B)
15 μm diameter

Figure 2-6b part 3
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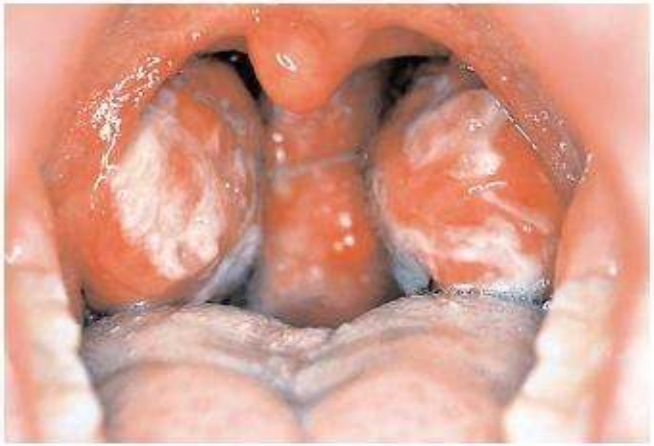
Mononucleosis



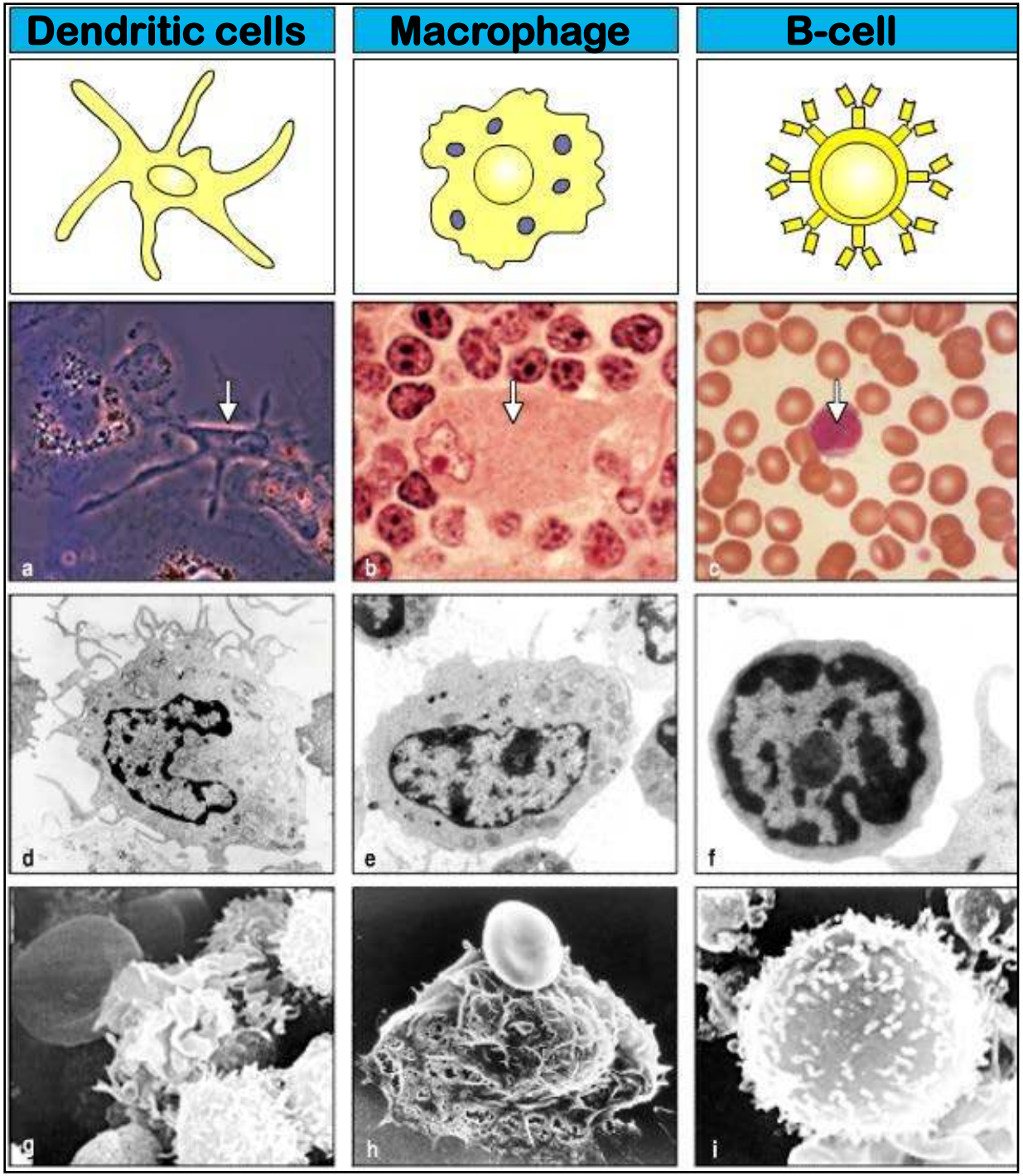
- Caused by Epstein-Barr virus
  - DNA herpes-types virus
- Infects 2 cell types
  - First epithelial cells of salivary gland- virus released in saliva
  - Then B lymphocytes via CD21
- Circulating B cells spread virus
  - to “reticuloendothelial system (liver, spleen, lymph nodes)
- Symptoms
  - Adenopathy, hepatosplenomegaly, fever, pharyngitis
  - Characteristic peripheral blood smear showing reactive lymphocytes

Antigen Presenting Cells

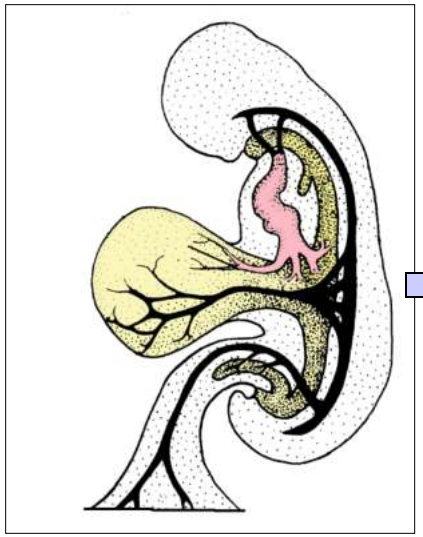
3 kinds of cells present Ag to T-cells

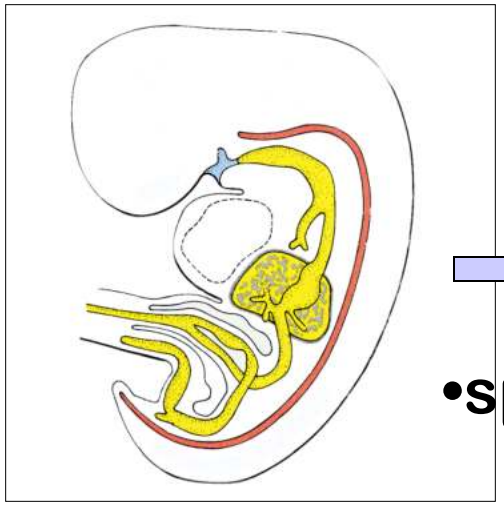
Dendritic cells: Several types

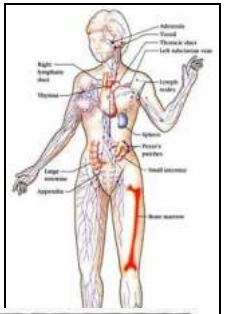
Capture, process, present Ag

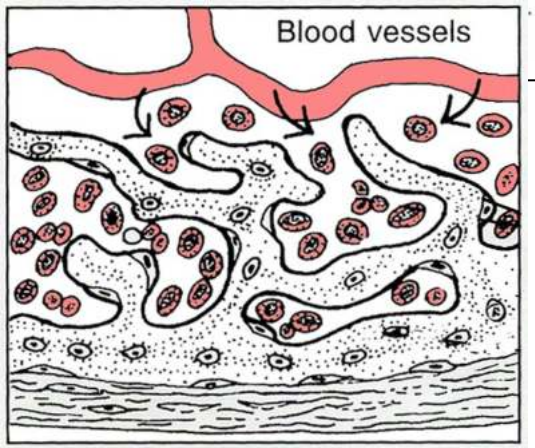


Organs of Hematopoesis…









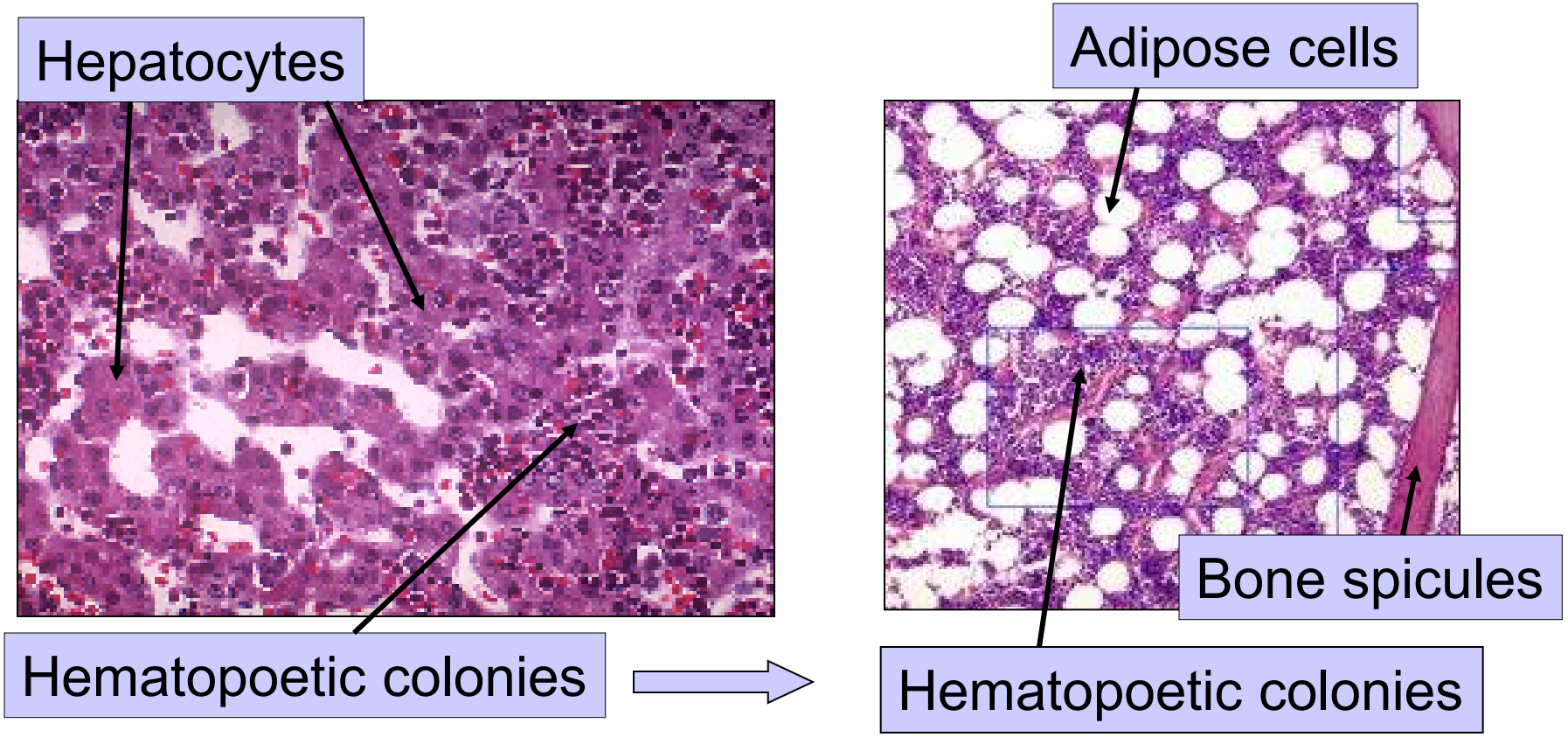
•spleen

Yolk Sac
- •3 weeks
- •Blood islands
- •Erythro-myeloid stem cells
- •RBC's are large and nucleated=*primitive*
- •*Cannot form lymphoid progeny*

Fetal Liver
- •5-6 weeks
- •Seeded from both outside sources
- •Max 6 mos then declines to neonatal stage

Bone Marrow
- •Source of all stem cells in adult
- •B-cell maturation
- •T-cells to thymus

Organs of Hematopoesis



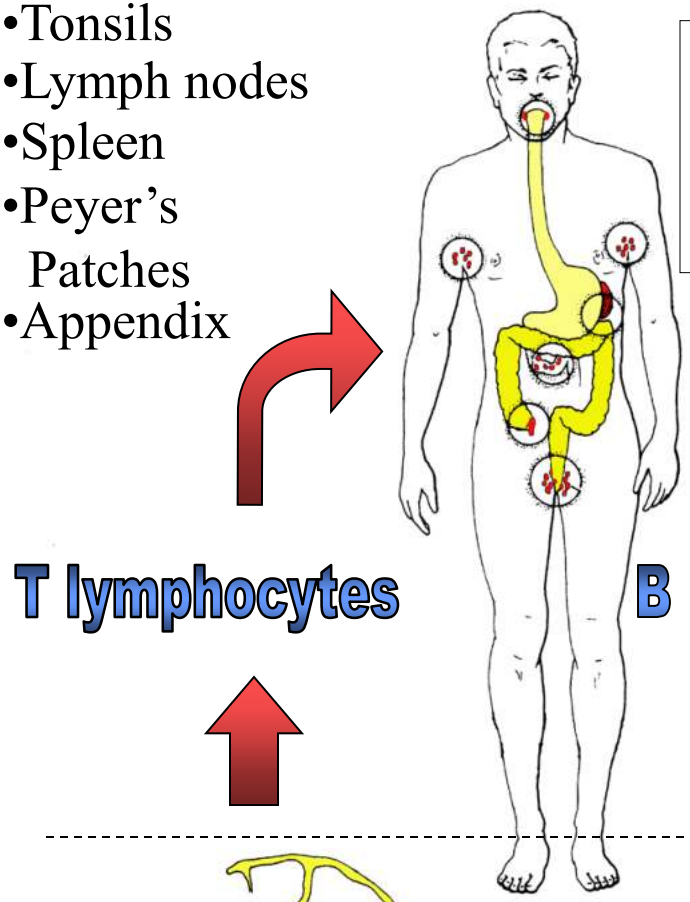
Fetal Liver

Bone Marrow

Organs of the Immune System

- Tonsils
- Lymph nodes
- Spleen
- Peyer's Patches
- Appendix

2. Distribution to Secondary lymphoid organs for engagement with antigens



T lymphocytes

B lymphocytes

Thymus

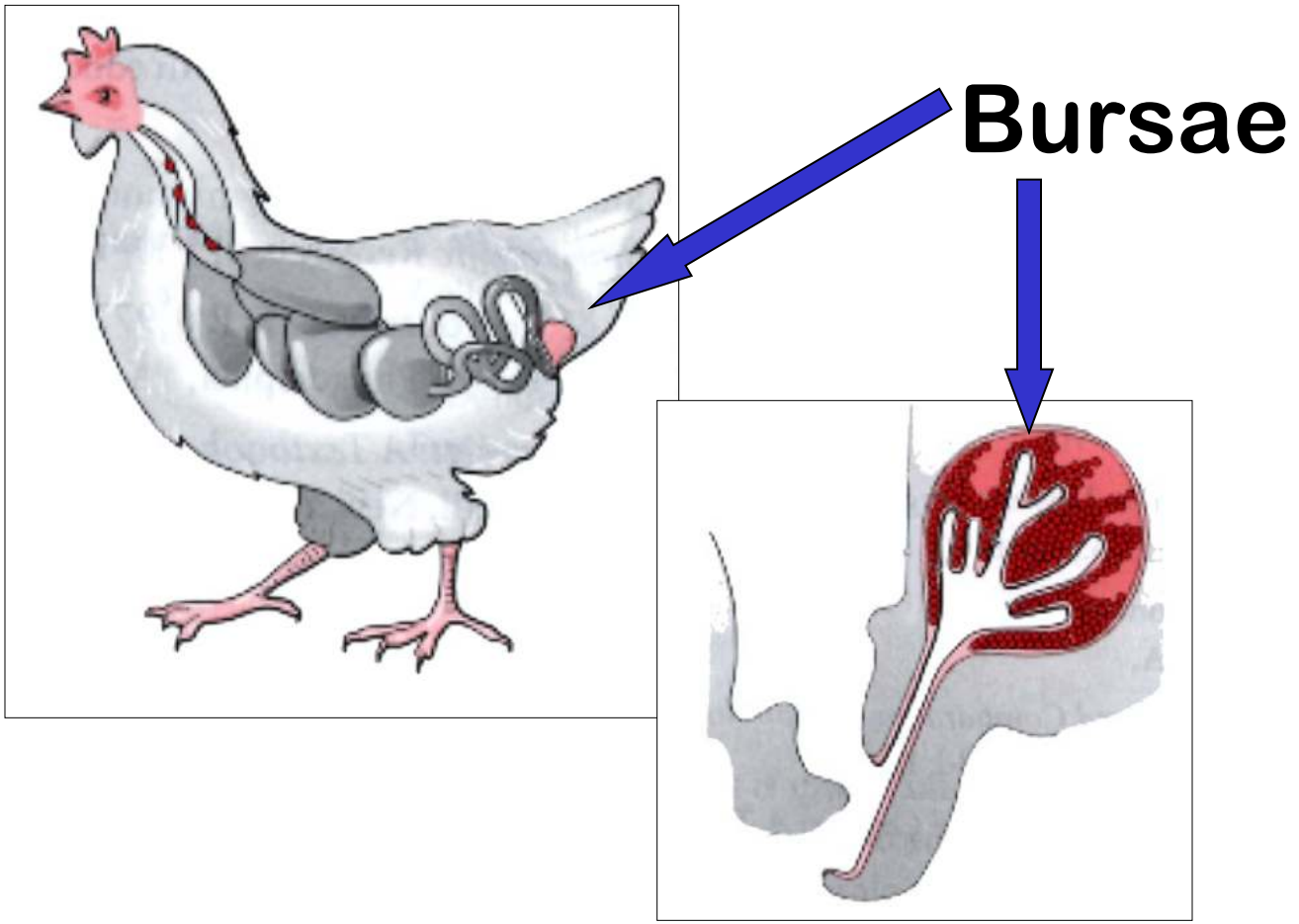
Bone Marrow

1. Development & maturation in primary lymphoid organs

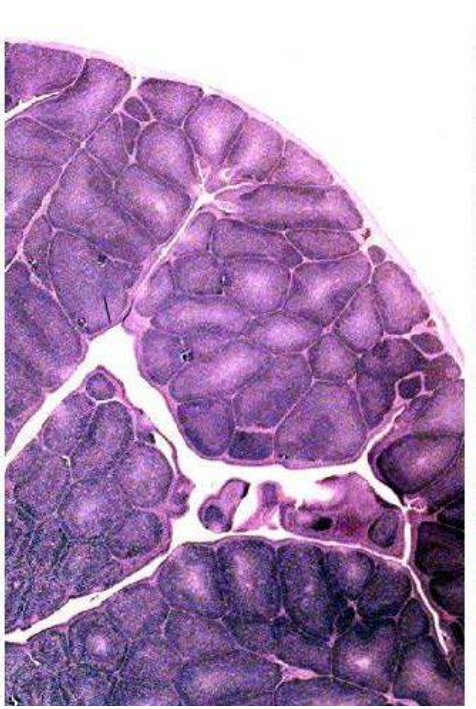
Stem cell
(in bone marrow)

In birds, the *Bursae of Fabricius* is the site of B-cell maturation

- Outpocketing of cloaca day 4-5
- Day 11-12, nodules form from lining: cortex and medulla



Bursae of Fabricius



- ***In mammals***, **B-cell maturation occurs in fetal liver and *bone marrow* after birth**

Maturation of T cells-

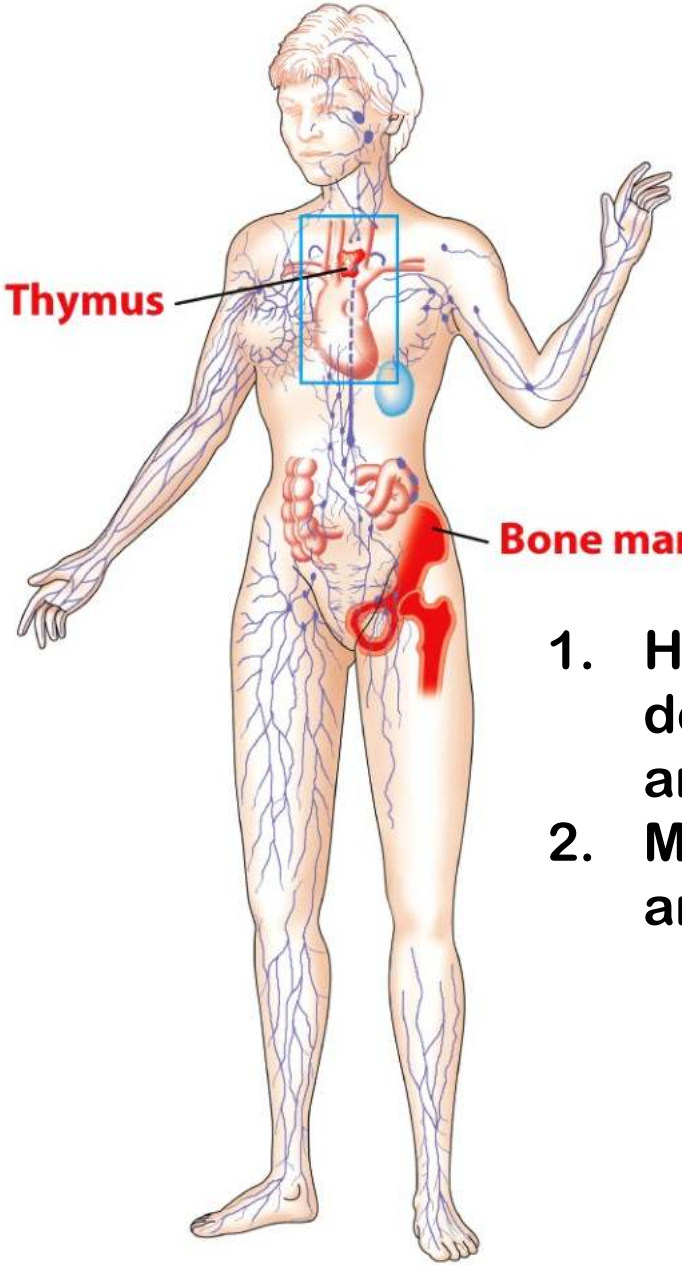


Figure 2-6a
Kuby Immunology, Seventh Edition

1. Hematopoesis/ development of myeloid and lymphoid cells
2. Maturation of myeloid and B-cells

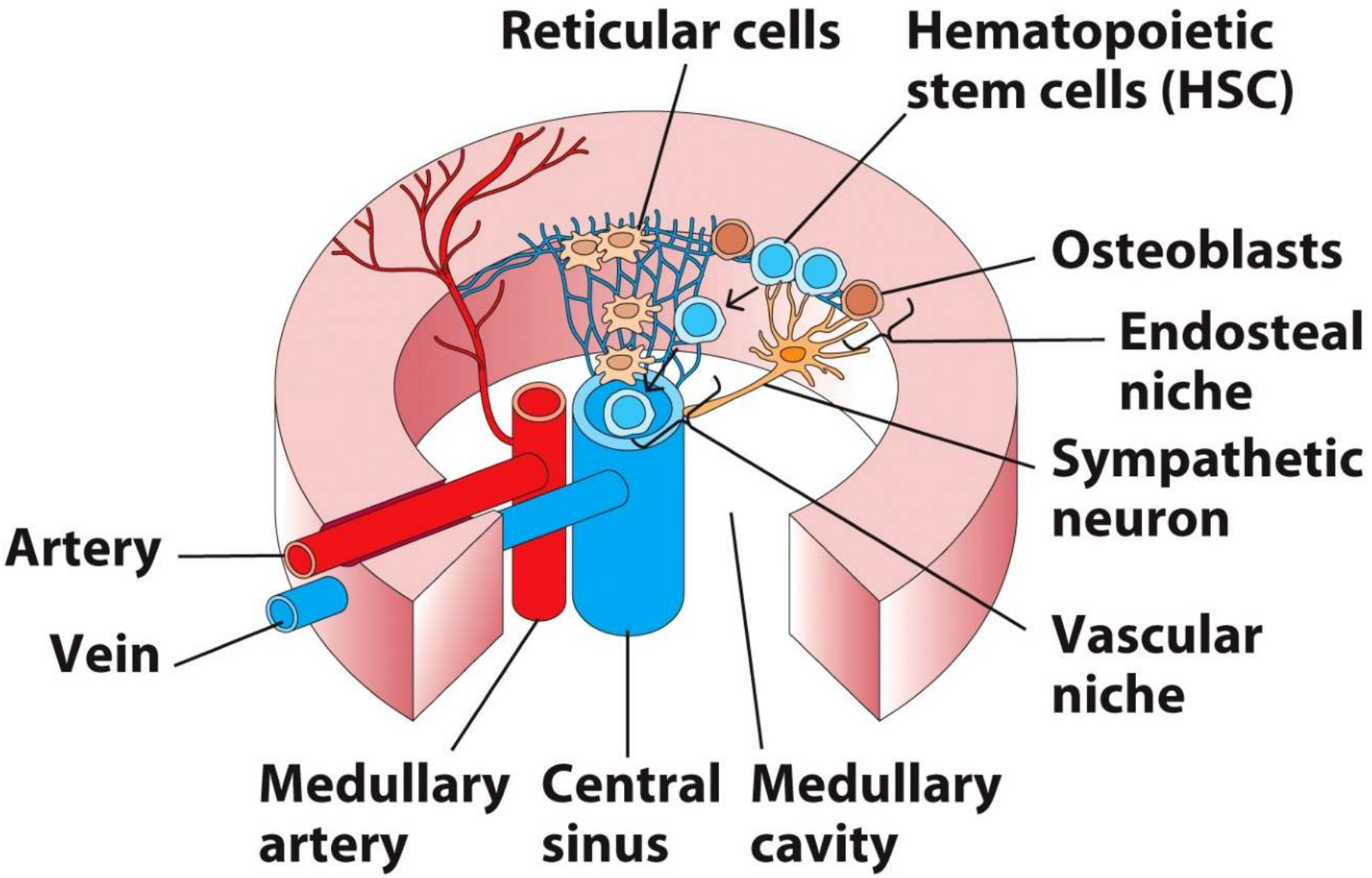
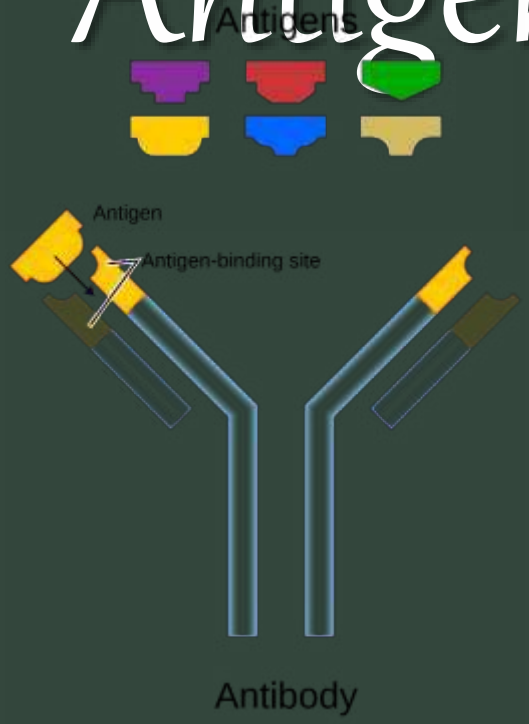
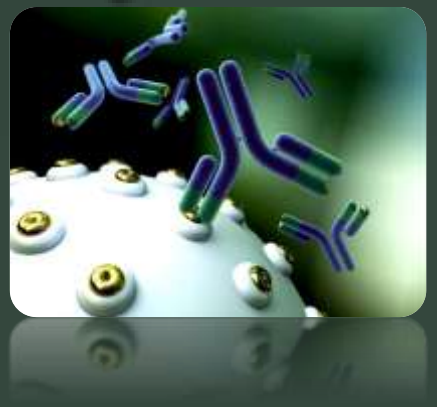


Figure 2-5c
Kuby Immunology, Seventh Edition


Good Afternoon

Antigen-Antibody System

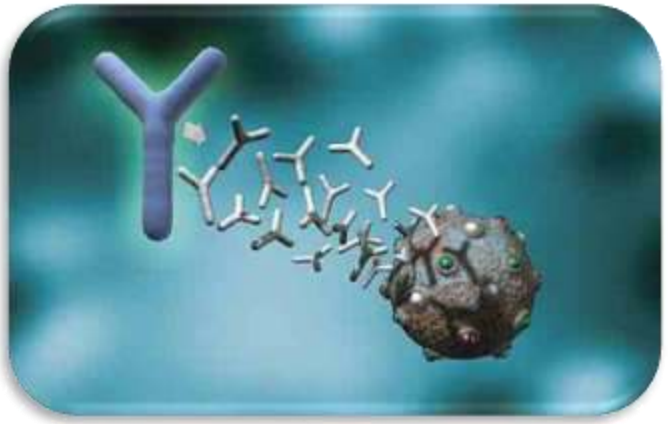




Dr. Nabeela Basha

Seminar - 5

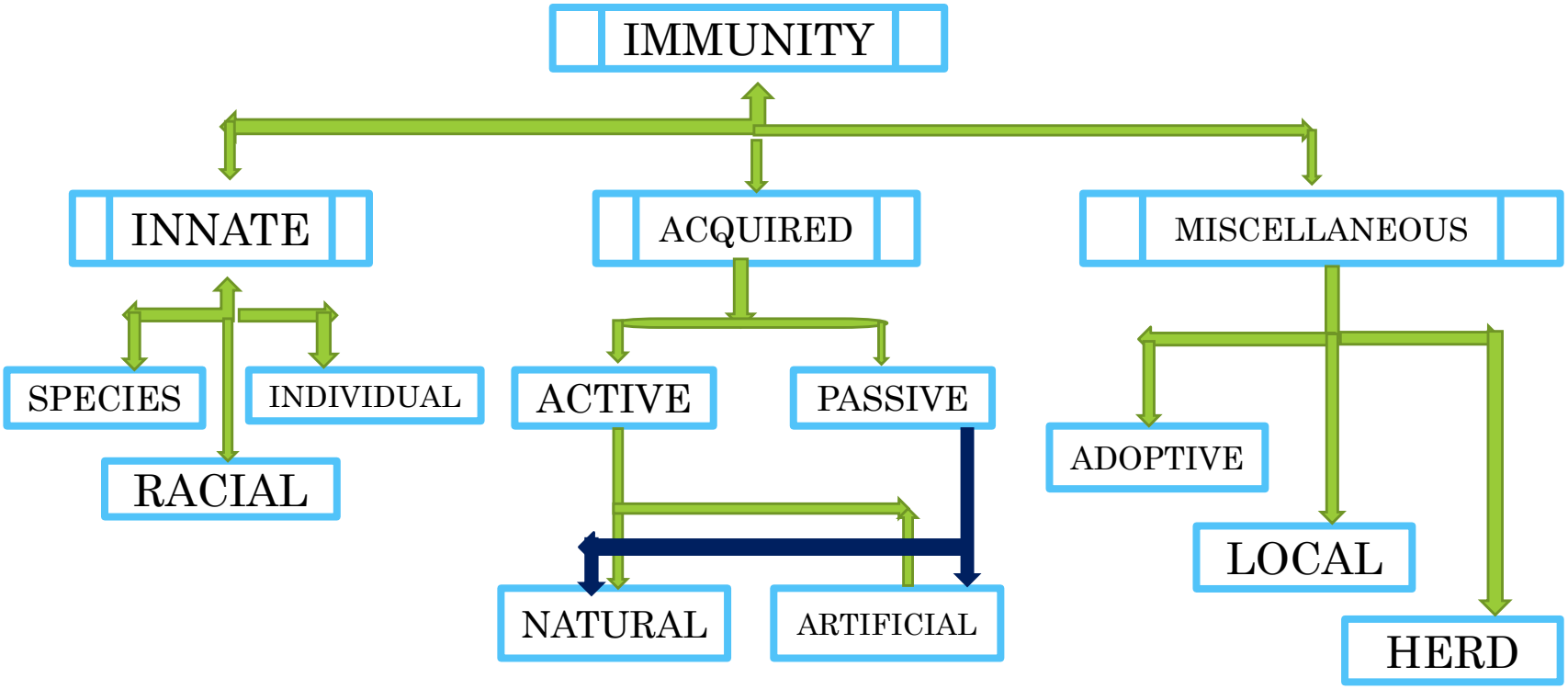
Contents

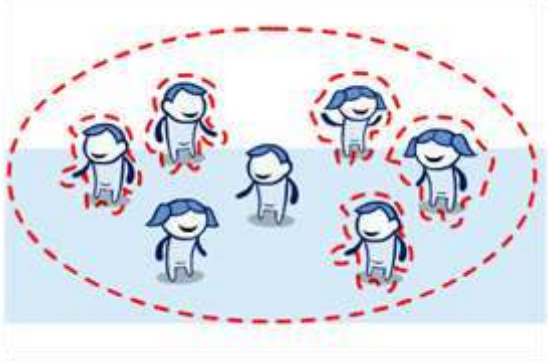


- Introduction
- Immunity
- Antigen
- Antibody
- Antigen antibody reactions
- Conclusion
- References
- Previous year questions

Introduction

- The term immunity is derived from immunitas (Latin for exemption from civic duties or paying taxes)
- The term ‘immunity’ is defined as resistance exhibited by the host against any foreign antigen including microorganisms.

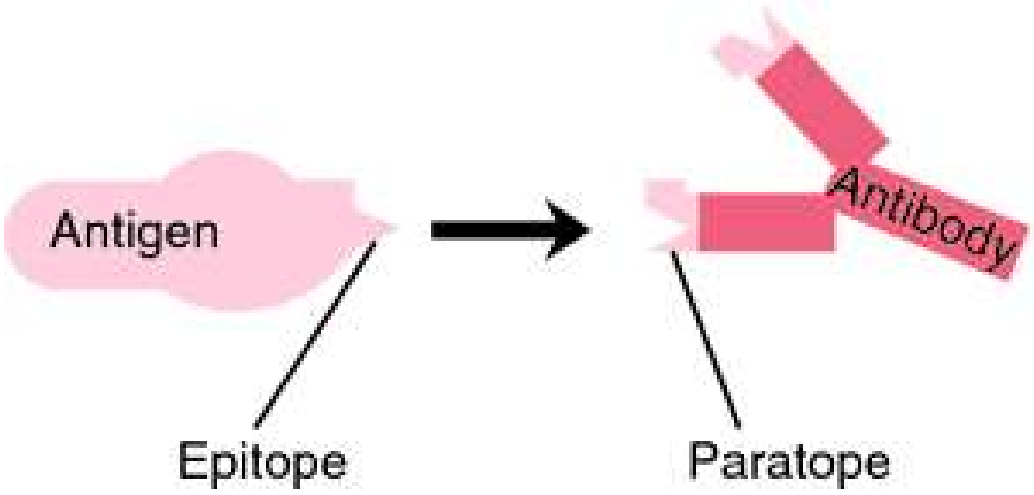

IMMUNITY
INNATE
ACQUIRED
MISCELLANEOUS
SPECIES
INDIVIDUAL
RACIAL
ACTIVE
PASSIVE
NATURAL
ARTIFICIAL
ADOPTIVE
LOCAL
HERD



Antigen

- An antigen is a substance which when introduced into a body evokes an immune response to produce a specific antibody with which it reacts specifically.
- It can be classified as-
  - Complete antigen
  - Incomplete antigen (Haptens)

- **Epitope** is the smallest unit of antigenicity.
- The combining site on the antibody molecule, corresponding to the epitope is called the **Paratope**.



Antibody

- These are substances which are formed in the serum and tissue fluids in response to an antigen and react with that antigen specifically and in some observable manner.
- Chemically they are globulins, hence they are named immunoglobulins.
- They constitute about 20 – 25% of the total serum proteins and are mainly synthesized by plasma cells.

Structure

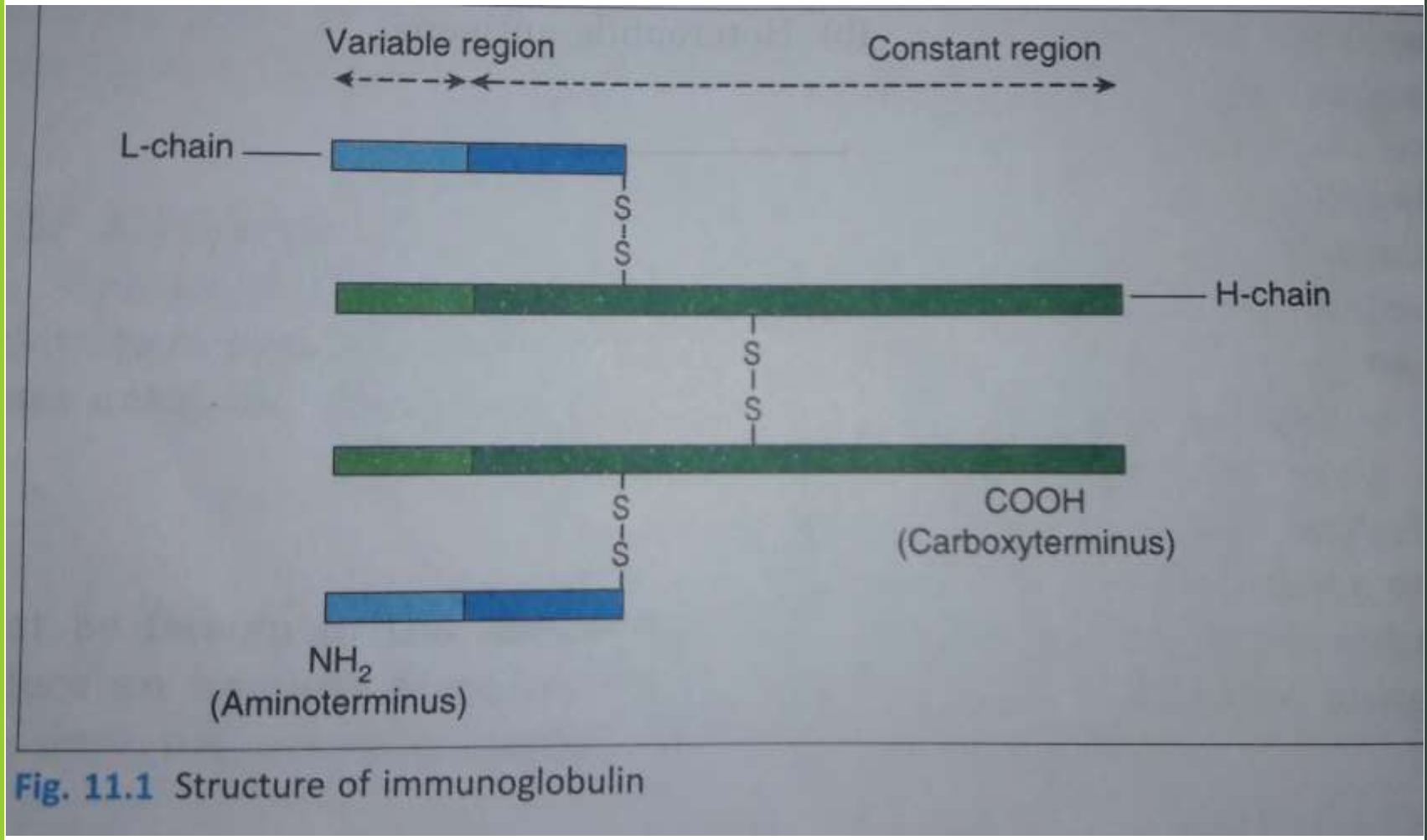


Fig. 11.1 Structure of immunoglobulin

Table 11.1: Properties of Immunoglobulin Classes

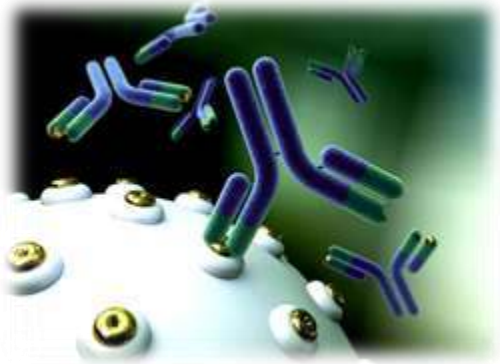
Property	IgG	IgA	IgM	IgD	IgE
Molecular weight	150,000	160,000	900,000	180,000	190,000
Sedimentation coefficient(S)	7	7	19	7	8
Heavy chain	Gamma	Alpha	Mu	Delta	Epsilon
Light chain	K or L	K or L	K or L	K or L	K or L
Serum concentration (mg/ml)	12	2	1.2	0.03	0.00004
Placental transport	+	–	–	–	–
Half life	23 days	6–8 days	5 days	3 days	2–3days
Intravascular distribution(%)	45	42	80	75	50
Present in milk	+	+	–	–	–

Role of Different Immunoglobulin Classes

IgG – protects the body fluids
IgA – protects the body surfaces
IgM – protects the blood stream
IgE – mediates reaginic hypersensitivity

Antigen Antibody Reactions

- The antigens and antibodies combine specifically with each other. This interaction between them is called Antigen – Antibody reaction.
- It may be abbreviated as Ag – Ab reaction.
- The first correct description of the antigen-antibody reaction was given by Richard J. Goldberg at the University of Wisconsin in 1952.



- These reactions form the basis for detection of infectious disease causing agents and also some non specific antigens like enzymes.
- The reactions between Ag and Ab occur in three stages.

- In first or *primary stage* the reaction involves formation of Ag-Ab complex.
- The *secondary stage* leads to visible events like precipitation, agglutination etc.
- The *tertiary stage* includes destruction of Ag or its neutralization.

Its USES are

1. **In vivo**

- Forms basis of immunity against infectious diseases.

2. **In vitro**

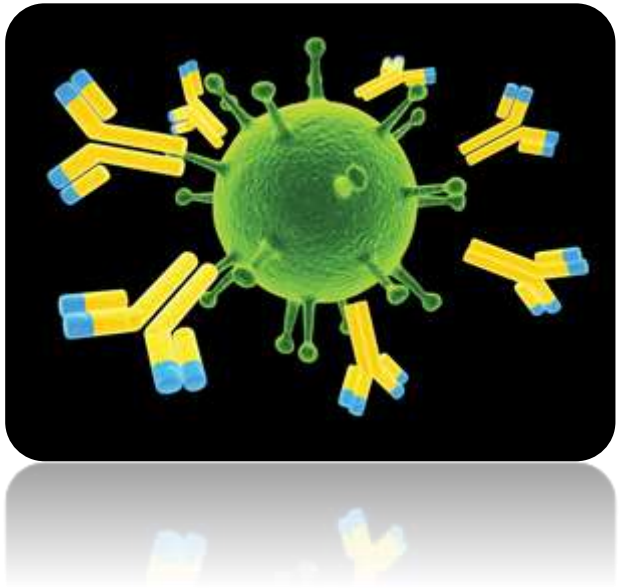
- For diagnosis of infections
- Helpful in epidemiological studies
- For identification of enzymes
- Detection and quantification of antigens or antibodies.

Characteristics

- Reaction is specific, an antigen combines only with its homologous antibody and vice-versa. However, cross reactions may occur due to antigenic similarity.
- Entire molecules of antigen and antibody react and not the fragments.
- Only the surface antigens participate in the antigen antibody reaction.
- The reaction is firm but reversible.

Types

- Precipitation reactions
- Agglutination
- Neutralization test
- Immunofluorescence
- Radioimmunoassay
- Enzyme linked immunosorbent assay
- Immunoelectronmicroscopic test



Precipitation reactions

- When a soluble antigen reacts with its antibody in the presence of electrolytes at an optimal temperature and pH, antigen antibody complex forms an insoluble precipitate that usually sediments at the bottom of the tube and it is called **precipitation**.

- Precipitation may occur in liquid media or in gels such as agar, agarose etc.

Applications

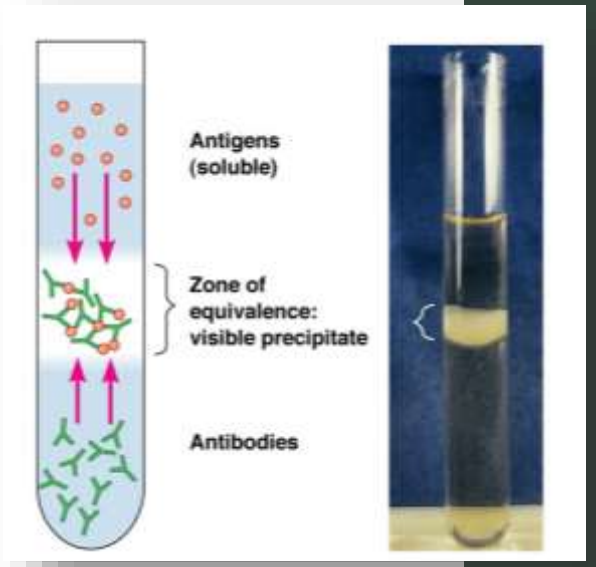
- Identification of bacteria. E.g., Lancefield grouping of streptococcus.
- Detection of antibody for diagnostic purposes. E.g., VDRL in syphilis
- Forensic application in identification of human blood and seminal stains
- To standardize toxins and antitoxins.

Types of precipitation reactions

- Ring test

e.g. C- reactive protein test

Streptococcal grouping by Lancefield technique

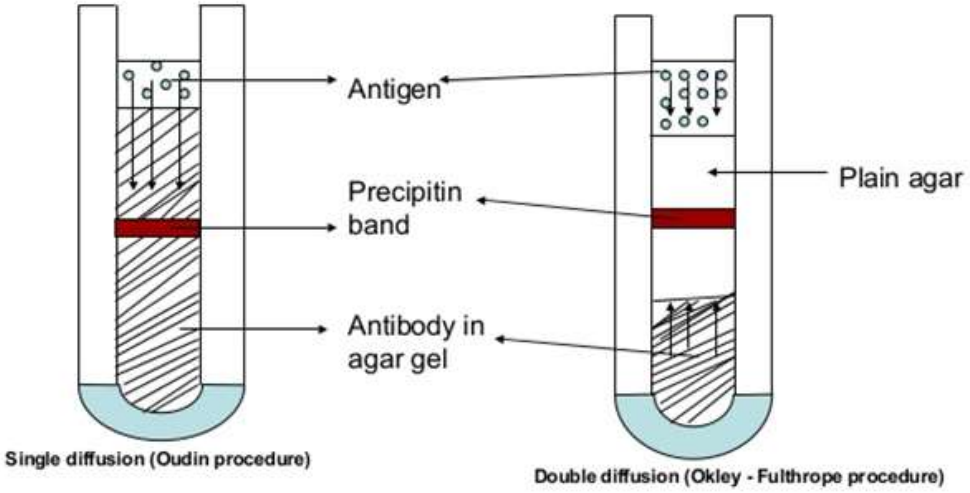


- Flocculation test

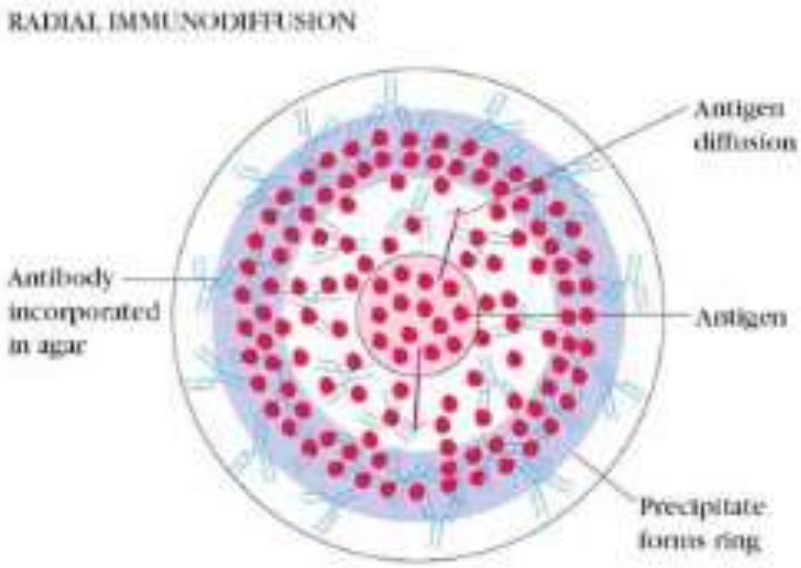
- Slide test. E.g., VDRL in syphilis

- Tube test. E,g., Kahn's test in syphilis

- Immunodiffusion test



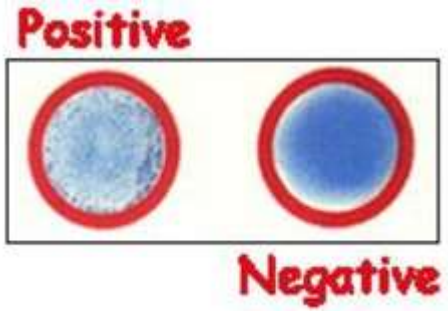
Single and double diffusion in one dimension



Agglutination

- It is an antigen antibody reaction in which a particulate antigen combines with its antibody in the presence of electrolytes at an optimal temperature and pH resulting in **visible clumping of particles**.
- It is more sensitive than precipitation for detection of antibodies.
- The reactions take place better with IgM antibody.

Types

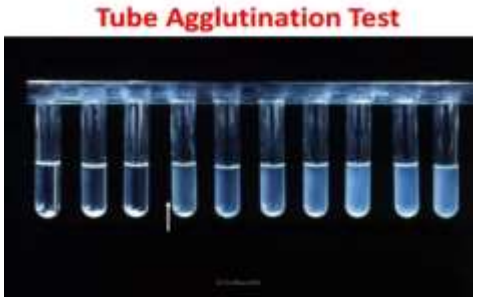


Slide Agglutination test

- Routine procedure to identify

bacterial stains. E.g., Salmonella species

- Also used for blood grouping.

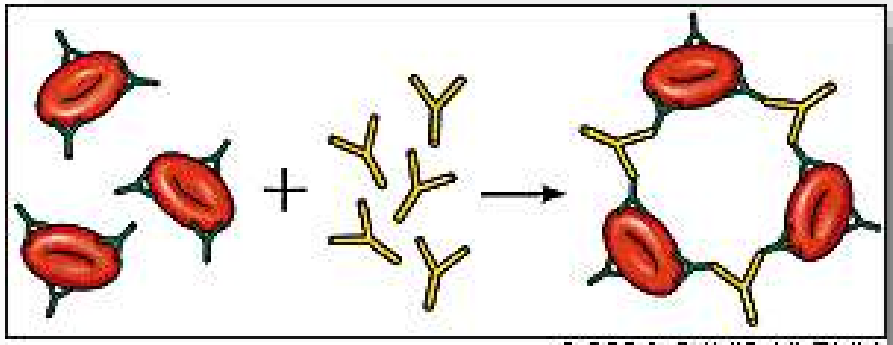


Tube Agglutination test

- Standard quantitative method for determination of antibodies.
- Routinely employed in diagnosis of typhoid, brucellosis and typhus fever

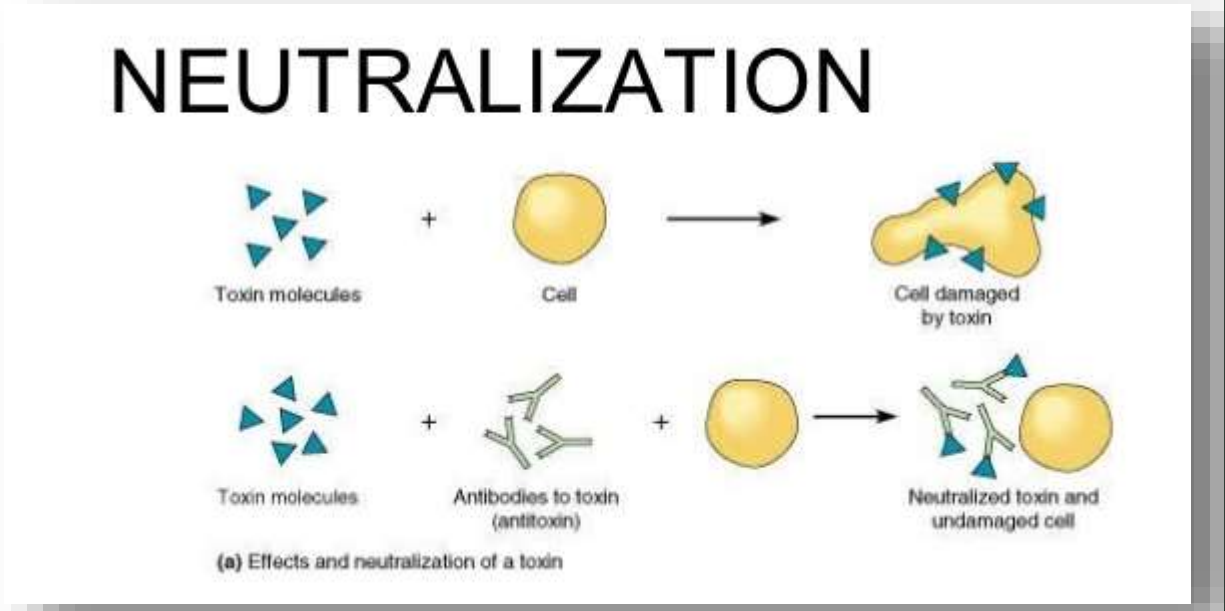
Coombs Test

- Originally devised by Coombs, Mourant and Race (1945) for detection of incomplete Rh antibodies.
- When sera containing incomplete anti-Rh antibodies are mixed with corresponding Rh-positive erythrocytes but no agglutination occurs.
- When such erythrocytes are treated with antiglobulin or COOMBS serum (rabbit antiserum with human gamma globulin), the cells are agglutinated.



Neutralization Test

- Bacterial exotoxins are capable of producing neutralizing antibodies (antitoxins) which play protective role in diseases such as diphtheria and tetanus.
- Toxin – antitoxin neutralization can be measured in vivo and in vitro.



In vivo tests:

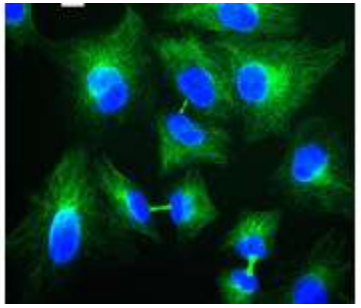
- Toxigenicity test. E.g., C. diphtheriae
- Shick test (similar test in human)

In vitro test:

- Antistreptolysin ‘O’ (ASO) test. E.g., Strep pyogenes
- Virus neutralization tests.

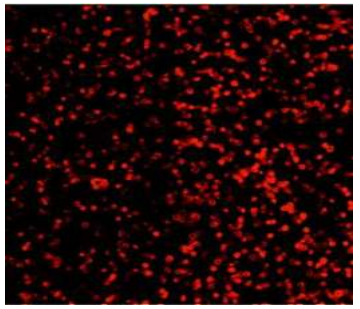
Immunofluorescence

- Fluorescence is the property of certain dyes which absorb rays of one particular wavelength (ultraviolet light) and emit rays with a different wavelength (visible light)



- Most commonly used dyes are:

1. Fluorescin isothiocyanate – blue green

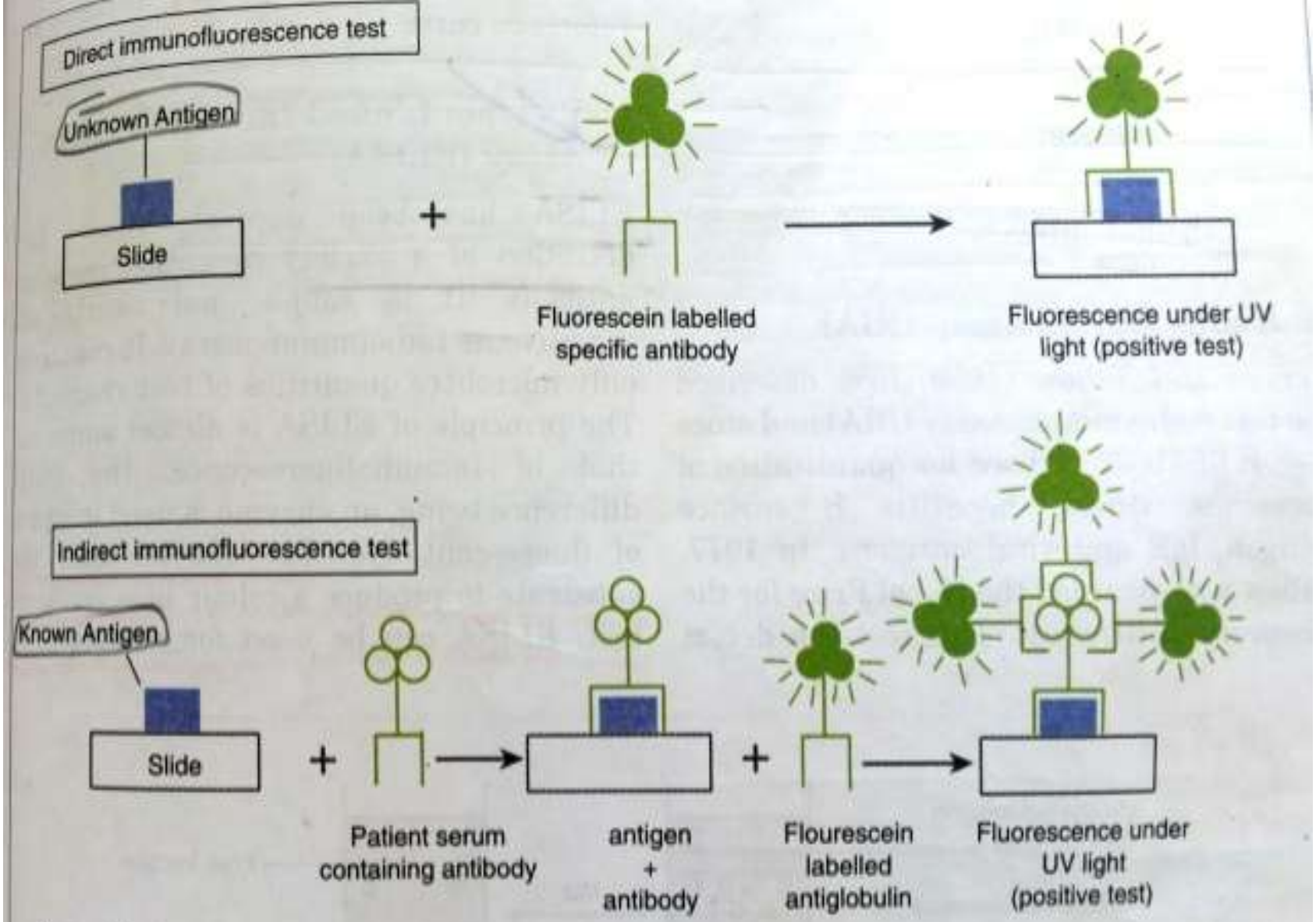


2. Lissamine rhodamine – orange red

- They are of two types:

1. Direct immunofluorescence

2. Indirect immunofluorescence



Example :

Treponema pallidum + Serum of syphilis patient (containing anti-treponemal antibodies which is globulin in nature) + Fluorescin labelled antiglobulin → Fluorescence (Positive)

Direct immunofluorescence

Uses:

- Commonly employed for detection of bacteria, viruses or other antigens in blood, urine, tissues and other specimens.
- Sensitive method to diagnosis Rabies.

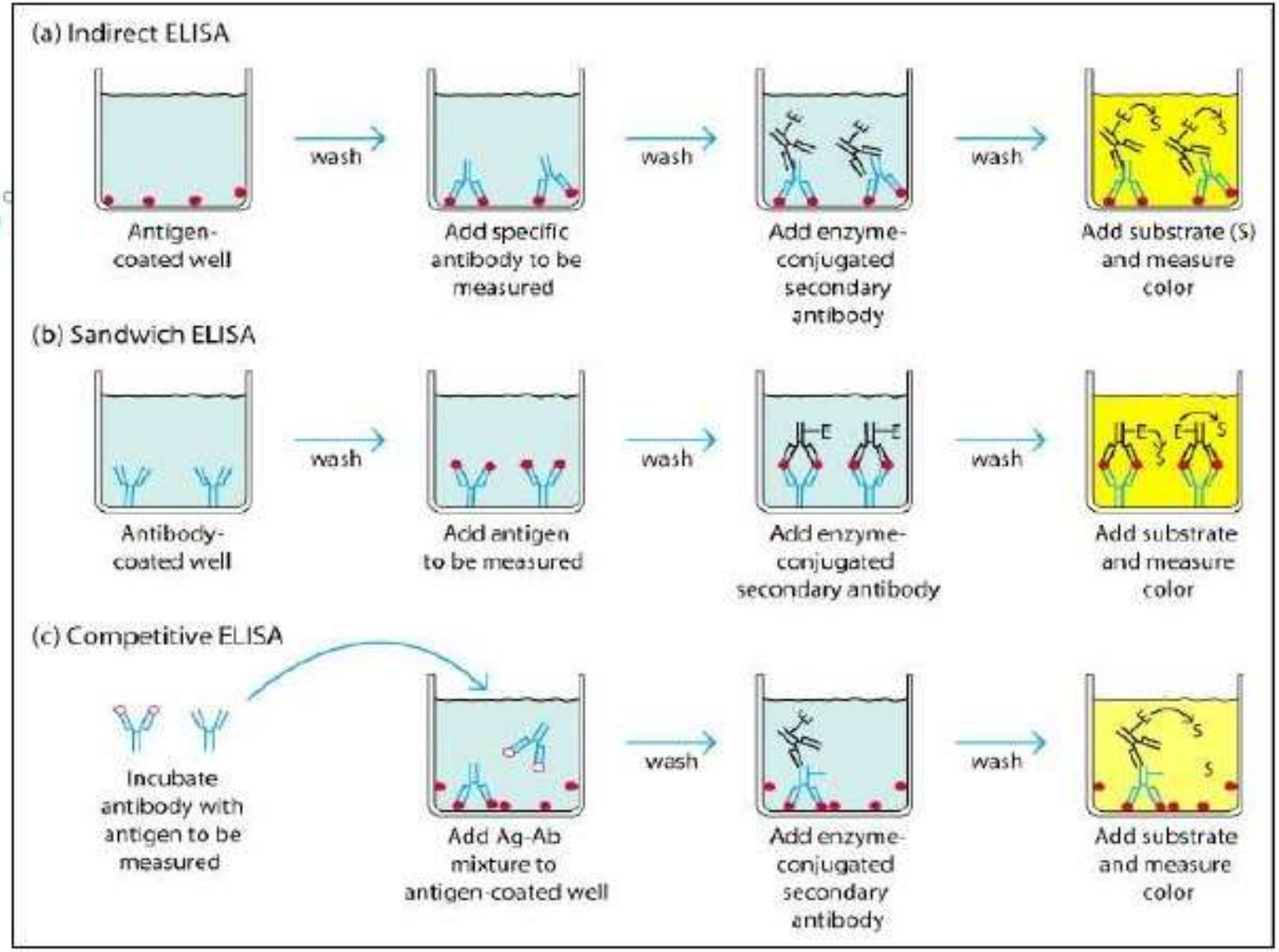
Disadvantage: Separate fluorescent labelled antibody has to be prepared for each antigen to be tested.

Indirect immunofluorescence

- A single antihuman globulin fluorescent conjugate can be employed for detection of antibody to any antigen
- This has overcome the disadvantage of direct immunofluorescence

ELISA

- Enzyme linked immunosorbent assay is a simple and a sensitive test.
- Requires only microlitre quantities of test reagents.
- The principle of ELISA is almost same as that of immunofluorescence, the only difference being, an enzyme is used instead of fluorescent dye.
- It can be used for detection of Antigen or Antibody.
- Types: Sandwich, Indirect, Competitive ELISA



Types of ELISA

Uses:

Detection of HIV antibodies in serum

Detection of mycobacterial antibodies in TB

Detection of Hepatitis B markers in serum

Detection of enterotoxin of E.coli in feces.

Immuno electron microscopic tests

1. Immunoelectron microscopy
2. Immuno Ferritin test
3. Immuno enzyme test

- Immuno electron microscopy: Viral particles are mixed with specific antisera and are observed under electron microscope. These are seen as clumps.

  Used in detection of Hepatitis A virus.

- Immuno ferritin test: Ferritin (electron dense substance) conjugated antibody is used to react with an antigen.

  Used in identification of Legionella species.

- Immuno enzyme test: Tissue sections are treated with peroxidase labelled antisera to detect the corresponding antigen and in viewed under electron microscope.

Conclusion

- Therefore we see the application of antigen antibody reactions in the diagnosis of diseases which can help in developments of varieties of diagnostic tests.
- In clinical practice, they help in:

- Preventing destructive diseases.
- Preventing progression of the diseases.
- Identifying high risk patients
- Target treatment of specific diseases
- Monitor the effects of the treatment.

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- Textbook of Microbiology for dental students. Dir. Prof. C. P Baveja. 4th edition
- Textbook of Microbiology for dental students. Ananthnarayan and Paniker. 8th edition
- Essential Microbiology for Dentistry, 4th Edition. Lakshman Samaranayake.
- https://en.wikipedia.org/wiki/Antigen_antibody_interaction

Previous year questions

- Antigen and Antibody system. (10marks) (MDS Degree Examination May 2009).

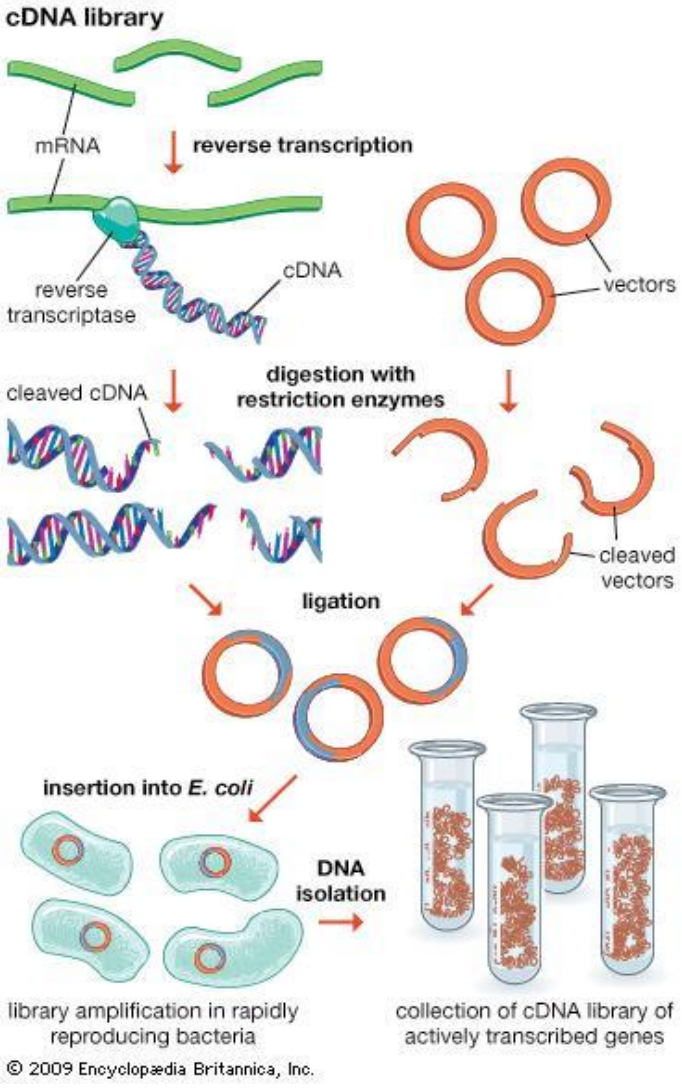

Thank you

Recombinant DNA technology

Recombinant DNA (rDNA) molecules are DNA molecules formed by laboratory methods of genetic recombination (such as molecular cloning) to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in biological organisms. Recombinant DNA is possible because DNA molecules from all organisms share the same chemical structure; they differ only in the sequence of nucleotides within that identical overall structure. Consequently, when DNA from a foreign source is linked to host sequences that can drive DNA replication and then introduced into a host organism, the foreign DNA is replicated along with the host DNA.

The steps involved in rDNA technology are:

1. Isolation of DNA
2. Fragmentation of the DNA using the enzyme Restriction endonucleases
3. Isolation of the desired DNA fragment
4. Amplification of the gene of interest
5. Ligation of the DNA fragment into a suitable vector by the enzyme DNA ligases
6. Transfer of DNA into the host cell
7. Screening
8. Culturing the host cells on a suitable medium on a large scale
9. Extraction of the desired product
10. Downstream processing of the products

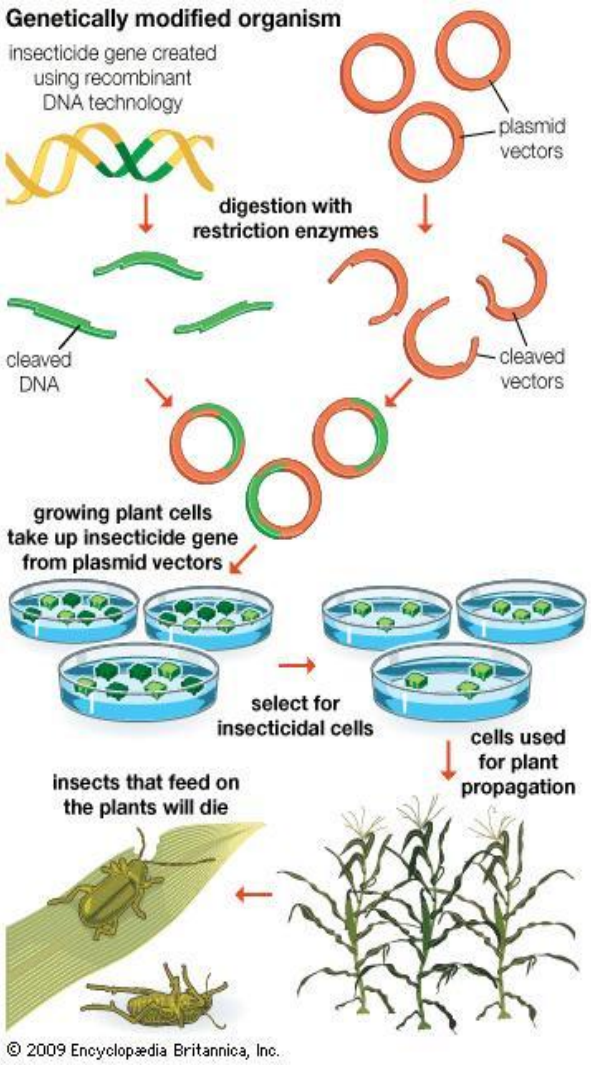


Insulin

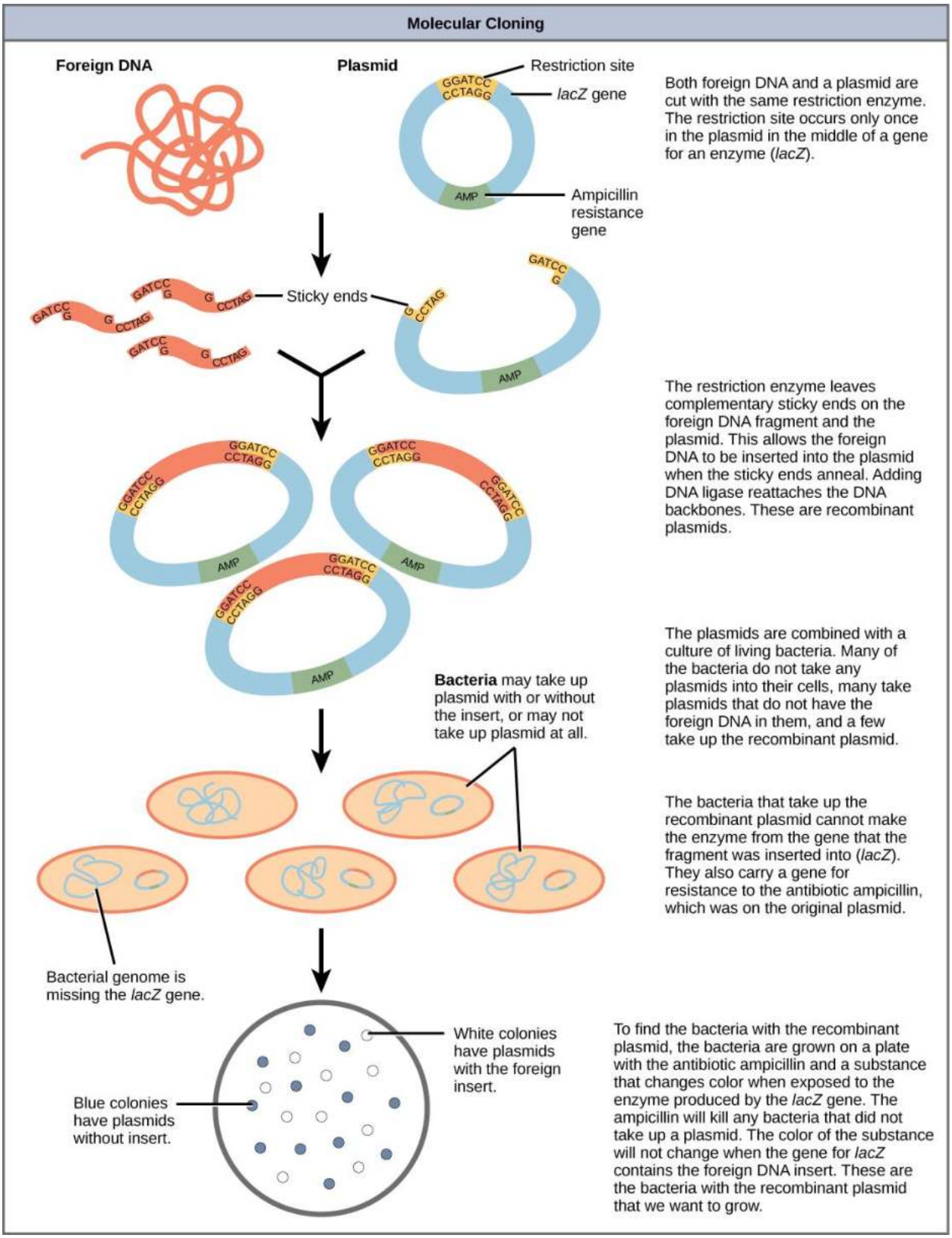
Insulin is a peptide hormone, produced by beta cells of the pancreas, and is central to regulating carbohydrate and fat metabolism in the body. The human insulin protein is composed of 51 amino acids, and has a molecular weight of 5808 Da. It is a dimer of an A-chain and a B-chain, which are linked together by disulfide bonds. The first genetically engineered, synthetic "human" insulin was produced in a laboratory in 1977 by Arthur Riggs, PhD and Keiichi Itakura, PhD at City of Hope and Herbert Boyer at Genentech using E. coli.

TPA (Tissue plasminogen activator)

Tissue plasminogen activator (abbreviated tPA or PLAT) is a protein involved in the breakdown of blood clots. It is a serine protease (EC 3.4.21.68) found on endothelial cells, the cells that line the blood vessels. As an enzyme, it catalyzes the conversion of plasminogen to plasmin, the major enzyme responsible for clot breakdown. Because it works on the clotting system, tPA is used in clinical medicine to treat embolic or thrombotic stroke. Use is contraindicated in hemorrhagic stroke and head trauma. tPA may be manufactured using recombinant biotechnology techniques. tPA created this way may be referred to as recombinant tissue plasminogen activator (rtPA).

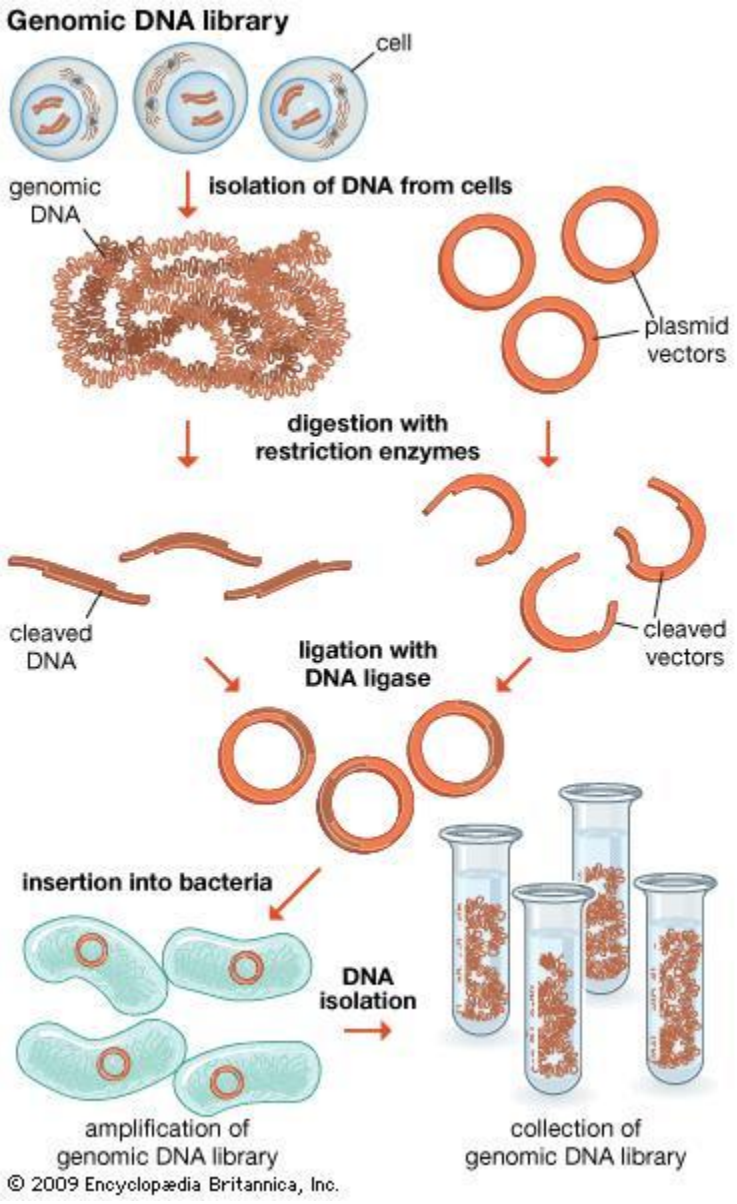


tPA is used in some cases of diseases that feature blood clots, such as pulmonary embolism, myocardial infarction, and stroke, in a medical treatment called thrombolysis. The most common use is for ischemic stroke. It can either be administered systemically, in the case of acutemyocardial infarction, acute ischemic stroke, and most cases of acute massive pulmonary embolism, or administered through an arterial catheter directly to the site of occlusion in the case of peripheral arterial thrombi and thrombi in the proximal deep veins of the leg


Molecular Cloning
Foreign DNA
Plasmid
Restriction site
lacZ gene
Ampicillin resistance gene
Sticky ends
AMP
Both foreign DNA and a plasmid are cut with the same restriction enzyme. The restriction site occurs only once in the plasmid in the middle of a gene for an enzyme (lacZ).
The restriction enzyme leaves complementary sticky ends on the foreign DNA fragment and the plasmid. This allows the foreign DNA to be inserted into the plasmid when the sticky ends anneal. Adding DNA ligase reattaches the DNA backbones. These are recombinant plasmids.
The plasmids are combined with a culture of living bacteria. Many of the bacteria do not take any plasmids into their cells, many take plasmids that do not have the foreign DNA in them, and a few take up the recombinant plasmid.
Bacteria may take up plasmid with or without the insert, or may not take up plasmid at all.
The bacteria that take up the recombinant plasmid cannot make the enzyme from the gene that the fragment was inserted into (lacZ). They also carry a gene for resistance to the antibiotic ampicillin, which was on the original plasmid.
Bacterial genome is missing the lacZ gene.
White colonies have plasmids with the foreign insert.
Blue colonies have plasmids without insert.
To find the bacteria with the recombinant plasmid, the bacteria are grown on a plate with the antibiotic ampicillin and a substance that changes color when exposed to the enzyme produced by the lacZ gene. The ampicillin will kill any bacteria that did not take up a plasmid. The color of the substance will not change when the gene for lacZ contains the foreign DNA insert. These are the bacteria with the recombinant plasmid that we want to grow.

Interferon's

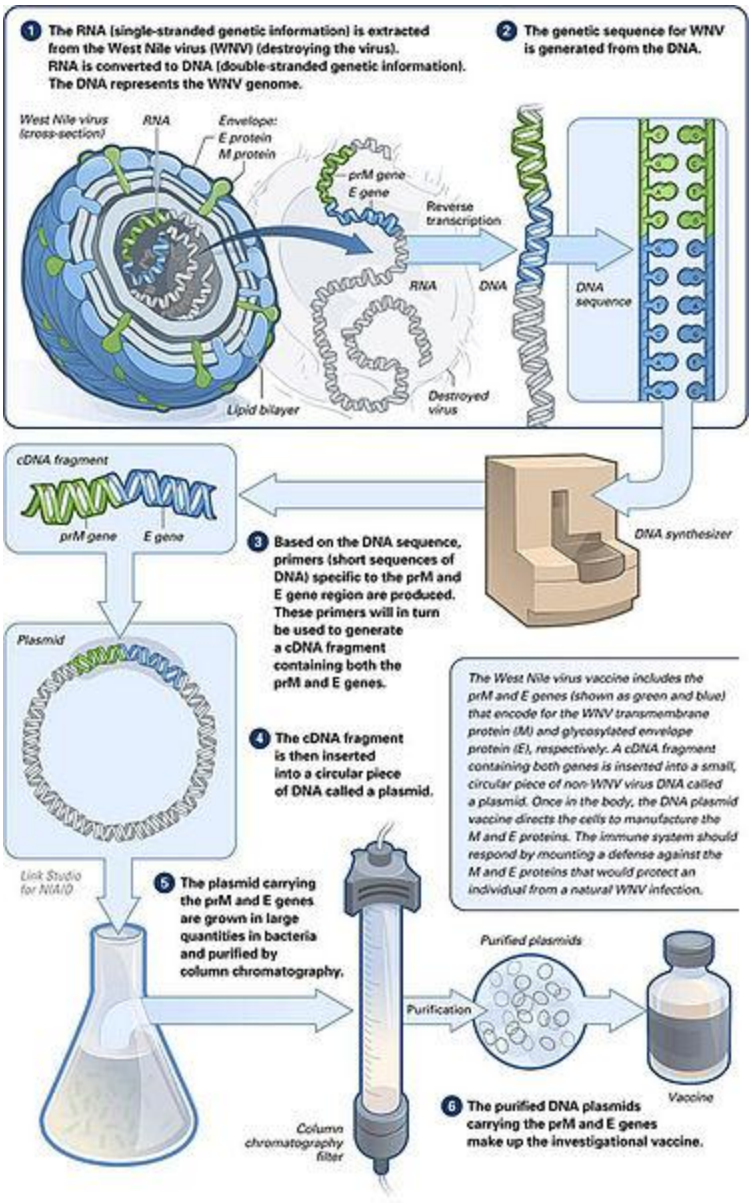
Interferon's (IFNs) are proteins made and released by host cells in response to the presence of pathogens such as viruses, bacteria, parasites or tumor cells. They allow for communication between cells to trigger the protective defenses of the immune system that eradicate pathogens or tumors.



IFNs belong to the large class of glycoproteins known as cytokines. Interferons are named after their ability to "interfere" with viral replication within host cells. IFNs have other functions: they activate immune cells, such as natural killer cells and macrophages; they increase recognition of infection or tumor cells by up-regulating antigen presentation to T lymphocytes; and they increase the ability of uninfected host cells to resist new infection by virus. Certain symptoms, such as aching muscles and fever, are related to the production of IFNs during infection.

About ten distinct IFNs have been identified in mammals; seven of these have been described for humans. They are typically divided among three IFN classes: Type I IFN, Type II IFN, and Type III IFN. IFNs belonging to all IFN classes are very important for fighting viral infections.

Interferon type I: All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains. The type I interferons present in humans are IFN-α, IFN-β and IFN-ω.



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, its toxins or one of its surface proteins. 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.

Vaccines may be prophylactic (example: to prevent or ameliorate the effects of a future infection by any natural or "wild" pathogen), or therapeutic (e.g. vaccines against cancer are also being investigated; see cancer vaccine).

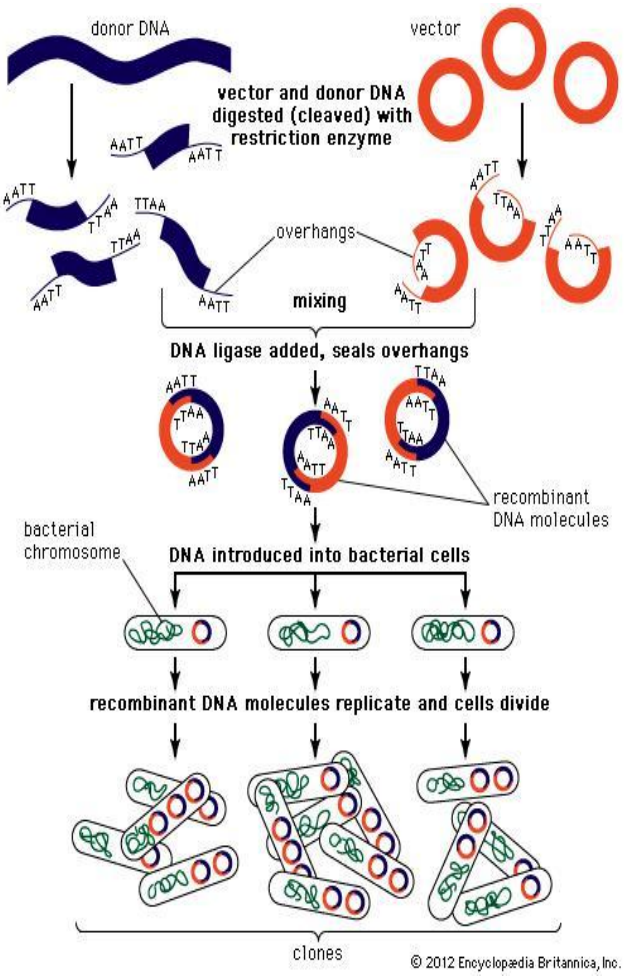
Recombinant vaccines:

Biotechnology sector has also played its part in developing vaccines against certain diseases. Such vaccine which makes use of recombinant DNA technology is known as recombinant vaccines. It is also known as subunit vaccines.

DNA vaccines:

DNA vaccination is a technique for protecting an organism against disease by injecting it with genetically engineered DNA to produce an immunological response. Nucleic acid vaccines are still experimental, and have been applied to a number of viral, bacterial and parasitic models of disease, as well as to several tumour models. DNA vaccines have a number of advantages over conventional vaccines, including the ability to induce a wider range of immune response types.

Here the gene encoding for immunogenic protein is isolated and used to produce recombinant DNA which acts as vaccine to be injected into the individual.



Steps involved:

Production of recombinant vaccines involves the following steps:

(i) First and foremost, it is important that the protein which is crucial to the growth and development of the causative organism be identified.

(ii) The corresponding gene is then isolated applying various techniques. Further to this, an extensive study of the gene explains the gene expression pattern involved in the production of corresponding protein.

(iii) This gene is then integrated into a suitable expression vector to produce a recombinant DNA.

(iv) This rDNA is used as vaccines or is introduce into another host organism to produce immunogenic proteins which acts as vaccines.

Human Growth Hormone

Human Growth Hormone, or HGH, is a hormone that is secreted from the pituitary gland (located at the base of your brain, near the front). It stimulates human growth and cell reproduction and regeneration.

As you age, your body naturally produces less HGH. You are no longer growing (like when you were a child or teenager) , so you no longer need to generate as many new cells on a regular basis.

And that's fine. But the loss of HGH is also what makes you age.

That's because HGH is not only responsible for skin cell growth; it also governs other bodily functions such as your metabolism, brain function, sexual responsiveness, ability to heal, muscle growth, weight loss, memory, and more.

Notice that all of the bodily functions governed by HGH are the areas that begin to suffer as you age.

For example, as HGH levels drop, your skin tone deadens, wrinkles appear, and skin slackens. That's because you no longer have the skin cell regeneration power of your youth.

As HGH levels continue to drop, you don't sleep as well anymore. You notice a decrease in your sex drive. That is a direct result of shifting hormone levels, plus changes in blood pressure and skin sensitivity - all of which are governed by HGH.

... Your muscle tone diminishes as your body begins to store more fat. This is due to decreased cell production and a reduced metabolic rate.

... Your clarity of thought and memory begin to suffer. Again, this is due to the slower rejuvenation of cells, this time in your brain.

The fact is, HGH directly and indirectly affects almost every part of your body.

Details of Steps involved in rDNA technology

1. Isolation of DNA - Genomic DNA, cDNA, Chemically Synthesis
2. Fragmentation of the DNA using the enzyme Restriction endonucleases
3. Isolation of the desired DNA fragment
4. Isolation of Vector - EColi Bactria (Plasmid PBR, PUC etc, Cosmid, Phage, YAC, BAC etc)
5. Amplification of the gene of interest
6. Ligation of the DNA fragment into a suitable vector by the enzyme DNA ligases
7. Transfer of DNA into the host cell – transformation, transduction, electroporation, liposome fusion, etc
8. Culturing the host cells on a suitable medium on a large scale
9. Extraction of the desired product
10. Downstream processing of the products

Example – Insulin production

1. Isolation of DNA - cDNA,
2. Fragmentation of the DNA using the enzyme Restriction endonucleases – Eco R1
3. Isolation of the desired DNA fragment
4. Isolation of Vector - EColi Bactria (Plasmid PBR)
5. Amplification of the gene of interest – PCR method
6. Ligation of the DNA fragment into a suitable vector by the enzyme DNA ligases
7. Transfer of DNA into the host cell – transformation CaCl2 treatment
8. Screening of rDNA insulin – insertional inactivation method
9. Culturing the host cells on a suitable medium on a large scale
10. Extraction of the desired product
11. Downstream processing of the products

UNIT II - R DNA TECHNOLOGY

Introduction

Genetic engineering is a tool of biotechnology sophisticated and most advanced. Genetic engineering comprises multiple techniques for the **intentional manipulation** of genetic material (primarily deoxyribonucleic acid, or DNA) to alter, repair, or enhance form or function.

Genetic engineering, also called recombinant DNA technology, involves the group of techniques used to cut up and join together genetic material, especially DNA from different biological species, and to introduce the resulting hybrid DNA into an organism in order to form new combinations of heritable genetic material.

- Recombinant **DNA** technology refers to the joining together of DNA molecules from two different species that are inserted into a host organism to produce new genetic combinations that are of value to science, medicine, agriculture, and industry.
- Recombinant DNA (rDNA), on the other hand is the general name for a piece of DNA that has been created by the combination of at least two strands.
- They are DNA molecules formed by laboratory methods of genetic recombination (such as molecular cloning) to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in the genome.

Steps of Genetic Recombination Technology

1. Isolation of Genetic Material

- The first step in rDNA technology is to isolate the desired DNA in its pure form i.e. free from other macromolecules.
- Since DNA exists within the cell membrane along with other macromolecules such as RNA, polysaccharides, proteins, and lipids, it must be separated and purified which involves enzymes such as lysozymes, cellulase, chitinase, ribonuclease, proteases etc.
- Other macromolecules are removable with other enzymes or treatments. Ultimately, the addition of ethanol causes the DNA to precipitate out as fine threads. This is then spooled out to give purified DNA.

2. Restriction Enzyme Digestion

- ✓ Restriction enzymes act as **molecular scissors** that cut DNA at specific locations. These reactions are called **'restriction enzyme digestions'**.
- ✓ They involve the incubation of the purified DNA with the selected restriction enzyme, at conditions optimal for that specific enzyme.
- ✓ The technique '**Agarose Gel Electrophoresis'** reveals the progress of the restriction enzyme digestion.
- ✓ This technique involves running out the DNA on an agarose gel. On the application of current, the negatively charged DNA travels to the positive electrode and is separated out based on size. This allows separating and cutting out the digested DNA fragments.
- ✓ The vector DNA is also processed using the same procedure.

3. Amplification Using PCR

- ✓ Polymerase Chain Reaction or PCR is a method of making multiple copies of a DNA sequence using the enzyme – DNA polymerase in vitro.
- ✓ It helps to amplify a single copy or a few copies of DNA into thousands to millions of copies.
- ✓ PCR reactions are run on 'thermal cyclers' using the following components:
- ✓ Template – DNA to be amplified
- ✓ Primers – small, chemically synthesized oligonucleotides that are complementary to a region of the DNA.
- ✓ Enzyme – DNA polymerase
- ✓ Nucleotides – needed to extend the primers by the enzyme.
- ✓ The cut fragments of DNA can be amplified using PCR and then ligated with the cut vector.

4. Ligation of DNA Molecules

- ✓ The purified DNA and the vector of interest are cut with the same restriction enzyme.
- ✓ This gives us the cut fragment of DNA and the cut vector, that is now open.
- ✓ The process of joining these two pieces together using the enzyme 'DNA ligase' is 'ligation'.
- ✓ The resulting DNA molecule is a hybrid of two DNA molecules – the interest molecule and the vector. In the terminology of genetics this intermixing of different DNA strands is called recombination.
- ✓ Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and the technology is referred to as the recombinant DNA technology.

5. Insertion of Recombinant DNA Into Host

- ✓ In this step, the recombinant DNA is introduced into a recipient host cell mostly, a bacterial cell. This process is '***Transformation'.***
- ✓ Bacterial cells do not accept foreign DNA easily. Therefore, they are treated to make them 'competent' to accept new DNA. The processes used may be thermal shock, Ca^{++}ion treatment, electroporation etc.

6. Isolation of Recombinant Cells

- ✓ The transformation process generates a mixed population of transformed and non-trans- formed host cells.
- ✓ The selection process involves filtering the transformed host cells only.
- ✓ For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector is employed.
- ✓ For examples, PBR322 plasmid vector contains different marker gene (Ampicillin resistant gene and Tetracycline resistant gene. When pst1 RE is used it knock out Ampicillin resistant gene from the plasmid, so that the recombinant cell become sensitive to Ampicillin.

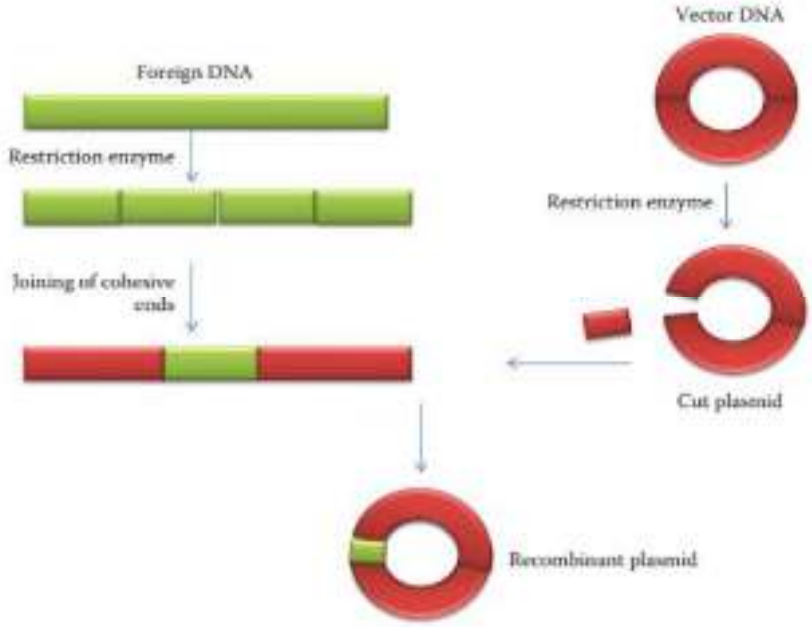


Figure 1: Making of recombinant DNA

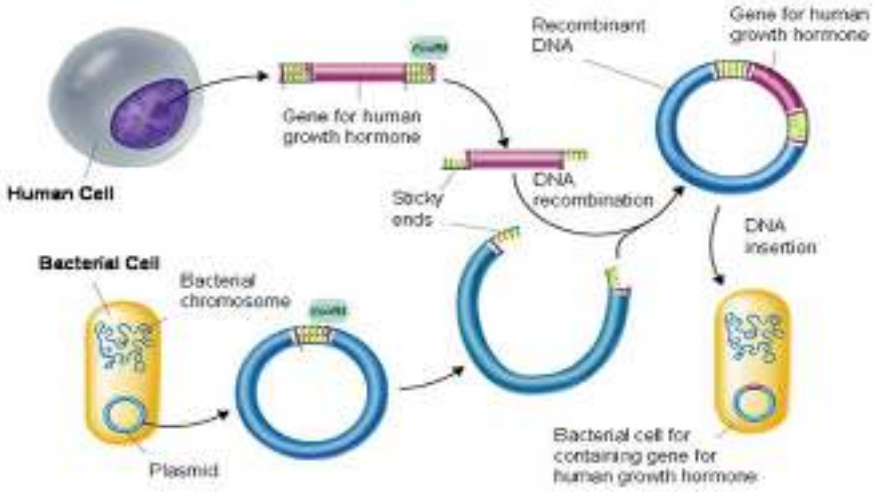


Figure 2: Process of recombinant DNA

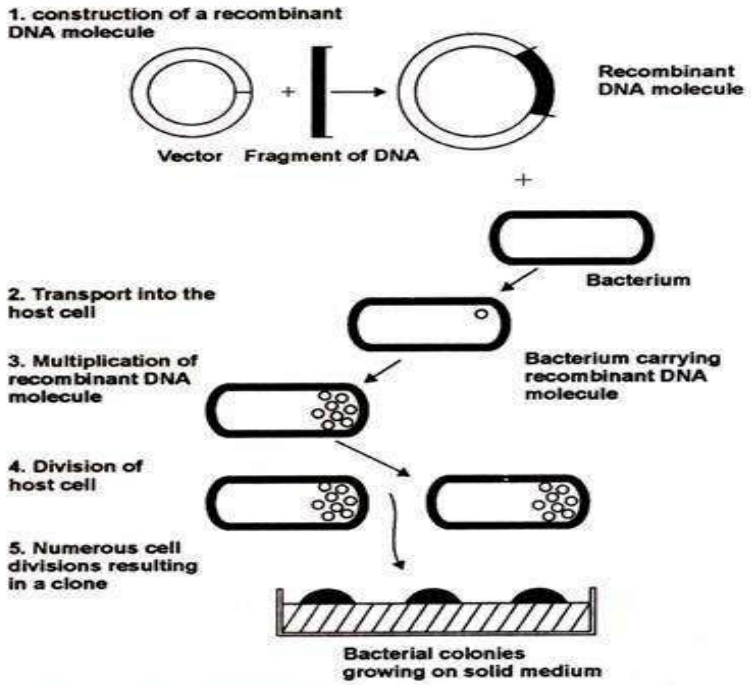


Fig. 1. The basic steps of rec DNA Technology using the bacterial plasmid as cloning vector.

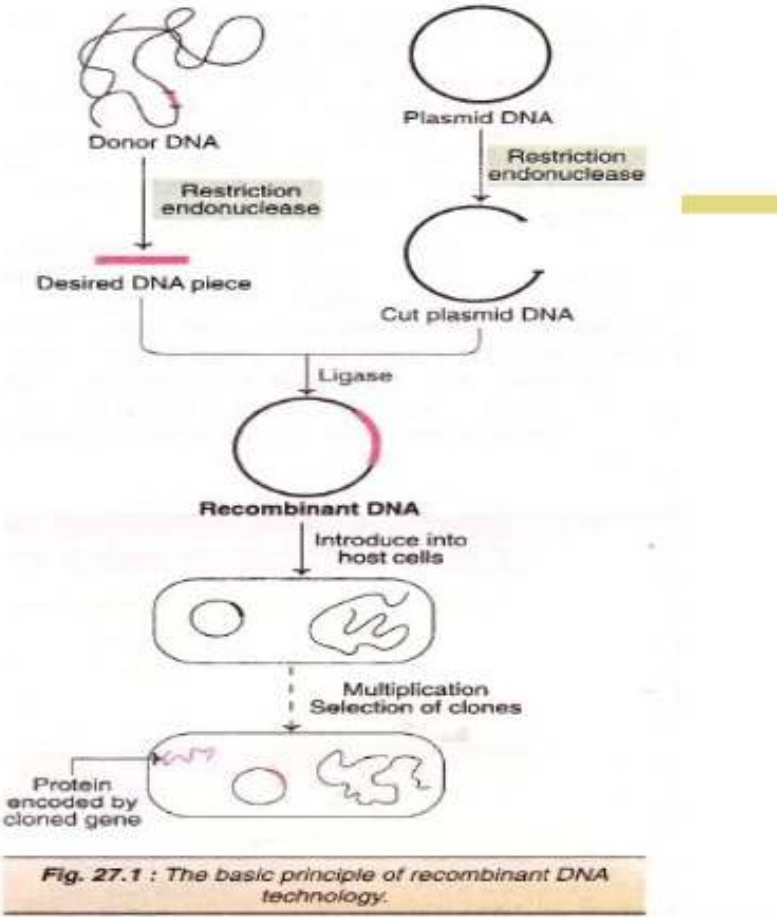


Fig. 27.1 : The basic principle of recombinant DNA technology.

STEPS in r DNA technology

i. Selection and isolation of DNA insert
ii. Selection of suitable cloning vector
iii. Introduction of DNA-insert into vector to form r DNA molecule
iv. r DNA molecule is introduced into a suitable host.
v. Selection of transformed host cells.
vi. Expression and multiplication of DNA-insert in the host.

(i) Selection and isolation of DNA insert:

First step in r DNA technology is the selection of a DNA segment of interest which is to be cloned. This desired DNA segment is then isolated enzymatically. This DNA segment of interest is termed as DNA insert or foreign DNA or target DNA or cloned DNA.

(ii) Selection of suitable cloning vector:

A cloning vector is a self-replicating DNA molecule, into which the DNA insert is to be integrated. A suitable cloning vector is selected in the next step of r DNA technology. Most commonly used vectors are plasmids and bacteriophages.

(iii) Introduction of DNA-insert into vector to form r DNA molecule:

The target DNA or the DNA insert which has been extracted and cleaved enzymatically by the selective restriction endonuclease enzymes [in step (i)] are now ligated (joined) by the enzyme ligase to vector DNA to form a r DNA molecule which is often called as cloning-vector-insert DNA construct.

(iv) r DNA molecule is introduced into a suitable host:

Suitable host cells are selected and the r DNA molecule so formed [in step (iii)] is introduced into these host cells. This process of entry of r DNA into the host cell is called *transformation.* Usually selected hosts are bacterial cells like *E. coli,* however yeast, fungi may also be utilized.

(v) Selection of transformed host cells:

Transformed cells (or recombinant cells) are those host cells which have taken up the r DNA molecule. In this step the transformed cells are separated from the non-transformed cells by using various methods making use of marker gene.

(vi) Expression and Multiplication of DNA insert in the host:

Finally, it is to be ensured that the foreign DNA inserted into the vector DNA is expressing the desired character in the host cells. Also, the transformed host cells are multiplied to obtain sufficient number of copies. If needed, such genes may also be transferred and expressed into another organism.

Tools for Recombinant DNA Technology:

r DNA technology utilizes a number of biological tools to achieve its objectives, most important of them being the enzymes.

Important biological tools for r DNA technology are:

(A) Enzymes:
- a. Restriction Endonucleases
- b. Exonucleases
- c. DNA ligases
- d. DNA polymerase

(B) Cloning Vector

(C) Host organism

(D) DNA insert or foreign DNA

(E) Linker and adaptor sequences.

An account of all these biological tools of genetic engineering is given below:

(A) ENZYMES:

A number of specific enzymes are utilized to achieve the objectives of r DNA technology.

The enzymology of genetic engineering includes the following types of enzymes:

(a) Restriction Endonuclease:

These enzymes serve as important tools to cut DNA molecules at specific sites, which is the basic need for r DNA technology.

These are the enzymes that produce internal cuts (cleavage) in the strands of DNA, only within or near some specific sites called recognition sites/recognition sequences/ restriction sites 01 target sites. Such recognition sequences are specific for each restriction enzyme. Restriction endonuclease enzymes are the first necessity for r DNA technology.

The ability to join DNA molecules together for cloning is dependent on the type of enzymes occurs in bacteria. These restriction enzymes are called restriction endonuclease or molecular scissors.

Restriction endonucleases are of bacterial origin and protect host cells as a weapon against foreign genes breaking them into smaller pieces and destroy them.

DNA converting into fragments of DNA

Types of Restriction Endonucleases:

There are 3 main categories of restriction endonuclease enzymes:

Type-I Restriction Endonucleases-

These are the complex type of endonucleases which cleave only one strand of DNA. These enzymes have the recognition sequences of about 15 bp length. This recognises a specific sequence but cuts the DNA outside the sequence 1000 base pairs away. They are most complex and not useful in gene manipulation as their cleavage sites are non specific. These enzymes with their subunits are responsible for methylation, endonucleolytic activity and site specificity.

Type-II Restriction Endonucleases-

These enzymes show cleavage in both the strands of DNA, are most important endonucleases for gene cloning. They show cleavage only at specific sites and therefore they produce the DNA fragments of a defined length. They cuts DNA within the recognisition sequence and are used for gene manipulation studies.

Type-III Restriction Endonucleases-

These are not used for gene cloning. They are the intermediate enzymes between Type-I and Type-II restriction endonuclease. They cuts the DNA approximately 25 base pairs away from recognisition sites.

Nature of cleavage by Restriction Endonucleases:

They cut the DNA molecule in two ways:

i. Many restriction endonucleases cleave both strands of DNA simply at the same point within the recognition sequence. As a result of this type of cleavage, the DNA fragments with blunt ends are generated.

ii. In the other style of cleavage by the restriction endonucleases, the two strands of DNA are cut at two different points. Such cuts are termed as staggered cuts and this results into the generation of protruding ends i.e., one strand of the double helix extends a few bases beyond the other strand. Such ends are, called cohesive or sticky ends.

(b) Exonucleases:

Exonuclease is an enzyme that removes nucleotides from the ends of a nucleic acid molecule. An exonuclease removes nucleotide from the 5′ or 3′ end of a DNA molecule. An exonuclease never produces internal cuts in DNA.

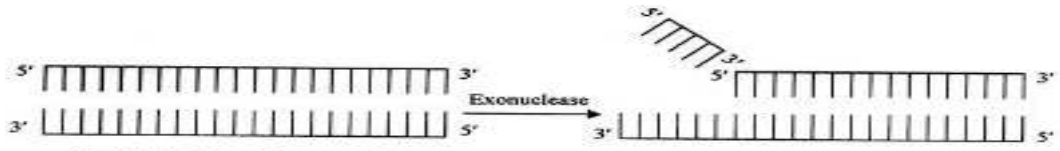


Fig. 4. An Exonuclease activity : Nucleotides are removed from the end of DNA

In r DNA technology, various types of exonucleases are employed like Exonuclease Bal 31, E. coli exonuclease III, Lambda exonuclease, etc.

(c) DNA ligase:

The function of these enzymes is to join two fragments of DNA by synthesizing the phosphodiester bond. They function to repair the single stranded nicks in DNA double helix and in r DNA technology they are employed for sealing the nicks between adjacent nucleotides. This enzyme is also termed as **molecular glue.**

(d) DNA polymerases:

These are the enzymes which synthesize a new complementary DNA strand of an existing DNA or RNA template. A few important types of DNA polymerases are used routinely in genetic engineering. One such enzyme is DNA polymerase ! which, prepared from E coli.

Examples:

DNA polymerase used in genetic engineering is Taq DNA polymerase which is used in PCR (Polymerase Chain Reaction).

Reverse transcriptase is also an important type of DNA polymerase enzyme for genetic engineering. It uses RNA as a template for synthesizing a new DNA strand called as c DNA a e complementary DNA). Its main use is in the formation of c DNA libraries.

Source and cleavage sites of restriction enzymes

Enzyme	Source	*Recognisition sequence*	*Cleavage site*
Hind III	*Haemophilus influenza*	5'AAGCTT 3'TTCGAA	5'---A AGCTT---3' 3'---TTCGA A---5'
Eco RI	*Escherichia coli*	5'GAATTC 3'CTTAAG	5'---G AATTC---3' 3'---CTTAA G---5'
BamHI	*Bacillus amyloliquefaciens*	5'GGATCC 3'CCTAGG	5'---G GATCC---3' 3'---CCTAG G---5'
SalI	*Streptomyces albus*	5'GTCGAC 3'CAGCTG	5'---G TCGAC---3' 3'---CAGCT G---5'
TaqI	*Thermus aquaticus*	5'TCGA 3'AGCT	5'---T CGA---3' 3'---AGC T---5'
Sau3AI	*Staphylococcus aureus*	5'GATC 3'CTAG	5'--- GATC---3' 3'---CTAG ---5'

(B) Cloning Vectors:

It is another important natural tool which geneticists use in r DNA technology. The cloning vector is the DNA molecule capable of replication in a host organism, into which the target DNA is introduced producing the r DNA molecule.

The cloning vector which has only a single site for cutting by a particular restriction endonuclease is considered as a good cloning vector. Different types of DNA molecules may be used as cloning vehicles such as they may be *plasmids, bacteriophages, cosmids, phasmids or artificial chromosomes.*

Features of Cloning Vectors

The cloning vectors possess the following features:

1. A cloning vector should possess an origin of replication so that it can self-replicate inside the host cell.
2. It should have a restriction site for the insertion of the target DNA.
3. It should have a selectable marker with an antibiotic resistance gene that facilitates screening of the recombinant organism.
4. It should be small in size so that it can easily integrate into the host cell.
5. It should be capable of inserting a large segment of DNA.
6. It should possess multiple cloning sites.
7. It should be capable of working under the prokaryotic and eukaryotic systems

Types of Cloning Vectors

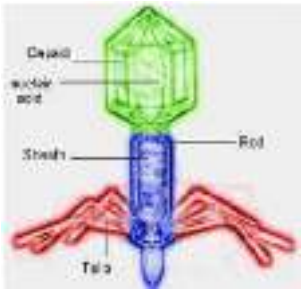
There are the following different types of cloning vectors:

Plasmids

- These were the first vectors used in gene cloning.
- These are found in bacteria, eukaryotes and archaea.

- These are natural, extra chromosomal, self-replicating DNA molecules.
- They have a high copy number and possess antibiotic-resistant genes.
- They encode proteins which are necessary for their own replication.
- *pBR322, pUC18, F-plasmid* are some of the examples of plasmid vectors

Bacteriophage

- These are more efficient than plasmids for cloning large DNA inserts.
- *Phage λ and M13 phage* are commonly used bacteriophages in gene cloning.
- 53 kb DNA can be packaged in the bacteriophage.
- The screening of phage plaques is much easier than the screening of recombinant bacterial colonies.
- 

Phagemids

- These are artificial vectors.
- They are used in combination with M13 phage.
- They possess multiple cloning sites and an inducible lac gene promoter.
- They are identified by blue-white screening.

Bacterial Artificial Chromosomes

- These are similar to *E. coli* plasmids vectors.
- It is obtained from naturally occurring F' plasmid.
- These are used to study genetic disorders.
- They can accommodate large DNA sequences without any risk.

Other cloning vectors include:

- Yeast Artificial Chromosomes
- Cosmids
- Retroviral Vectors
- Human Artificial Chromosomes

(C) Host Organism:

A good host organism is an essential tool for genetic engineering. Most widely used host for r DNA technology is the bacterium *E. coli.* because cloning and isolation of DNA inserts is

very easy in this host. A good host organism is the one which easy to transform and in which the replication of r DNA is easier. There should not be any interfering element against the replication of r DNA in the host cells.

(D) DNA Insert or Foreign DNA:

The desired DNA segment which is to be cloned is called as DNA insert or foreign DNA or target DNA. The selection of a suitable target DNA is the very first step of r DNA technology. The target DNA (gene) may be of viral, plant, animal or bacterial origin.

Following points must be kept in mind while selecting the foreign DNA:

- ✓ It can be easily extracted from source.
- ✓ It can be easily introduced into the vector.
- ✓ The genes should be beneficial for commercial or research point of view.

A number of foreign genes are being cloned for benefit of human beings. Some of these DNA inserts are the genes responsible for the production of insulin, interferon's, lymphotoxins various growth factors, interleukins, etc.

(E) Linker and Adaptor Sequences:

Linkers and adaptors are the DNA molecules which help in the modifications of cut ends of DNA fragments. These can be joined to the cut ends and hence produce modifications as desired. Different types of linkers and adaptors are used for different purposes.

Application of Recombinant DNA technology

r DNA is widely used in biotechnology, medicine & research

- ✓ The most common application of recombinant DNA is in basic research, in which the technology is important to most current work in the biological and biomedical sciences.
- ✓ Recombinant DNA is used to identify, map and sequence genes, and to determine their function.
- ✓ Recombinant proteins are widely used as reagents in laboratory experiments and to generate antibody probes for examining protein synthesis within cells and organisms.
- ✓ Many additional practical applications of recombinant DNA are found in industry, food production, human and veterinary medicine, agriculture, and bioengineering.
- ✓ DNA technology is also used to detect the presence of HIV in a person.
- ✓ Application of recombinant DNA technology in Agriculture – For example, manufacture of Bt-Cotton to protect the plant against ball worms.
- ✓ Application of medicines – Insulin production by DNA recombinant technology is a classic example.
- ✓ Gene Therapy – It is used as an attempt to correct the gene defects which give rise to heredity diseases.
- ✓ Clinical diagnosis – ELISA is an example where the application of recombinant DNA is possible.

Some recombinant DNA products being used in human therapy:

Using procedures like this, many human genes have been cloned in *E. coli* or in yeast. This has made it possible — for the first time — to produce unlimited amounts of human proteins in vitro. Cultured cells (*E. coli*, yeast, mammalian cells) transformed with a human gene are being used to manufacture more than 100 products for human therapy. Some examples:

- Insulin for diabetics
- Factor VIII for males suffering from hemophilia A
- Factor IX for hemophilia B
- Human growth hormone (HGH)
- Erythropoietin (EPO) for treating anemia
- Several types of interferons
- Several interleukins
- Granulocyte-macrophage colony-stimulating factor (GM-CSF) for stimulating the bone marrow after a bone marrow transplant
- Granulocyte colony-stimulating factor (G-CSF) for stimulating neutrophil production (e.g., after chemotherapy) and for mobilizing hematopoietic stem cells from the bone marrow into the blood.
- Tissue plasminogen activator (TPA) for dissolving blood clots
- Adenosine deaminase (ADA) for treating some forms of severe combined immunodeficiency (SCID)
- Parathyroid hormone
- Many monoclonal antibodies
- Hepatitis B surface antigen (HBsAg) to vaccinate against the hepatitis B virus
- C1 inhibitor (C1INH) used to treat hereditary angioedema

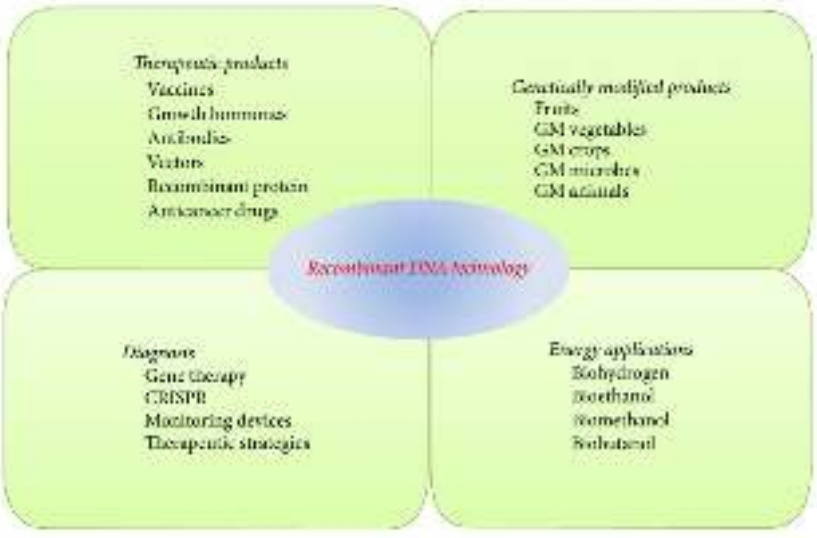
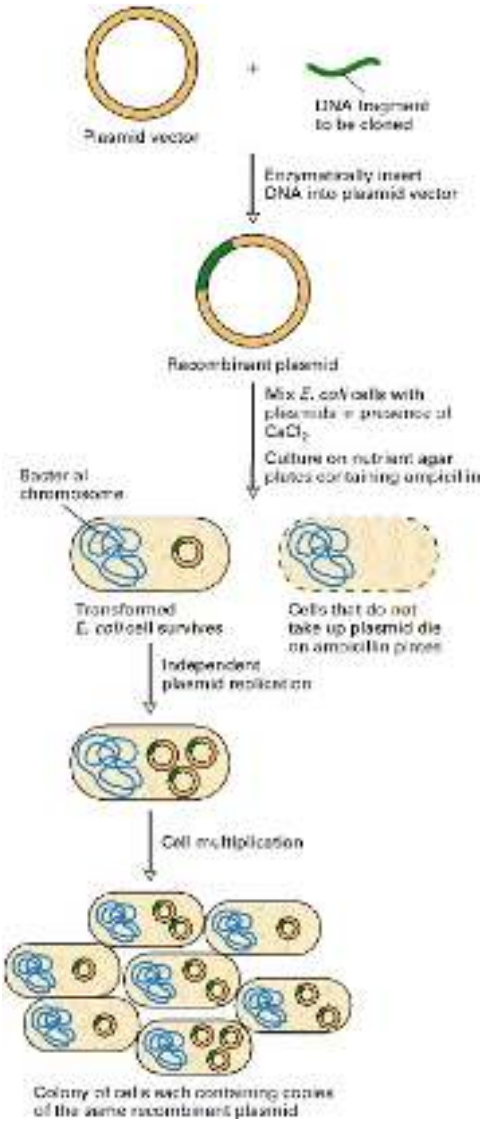
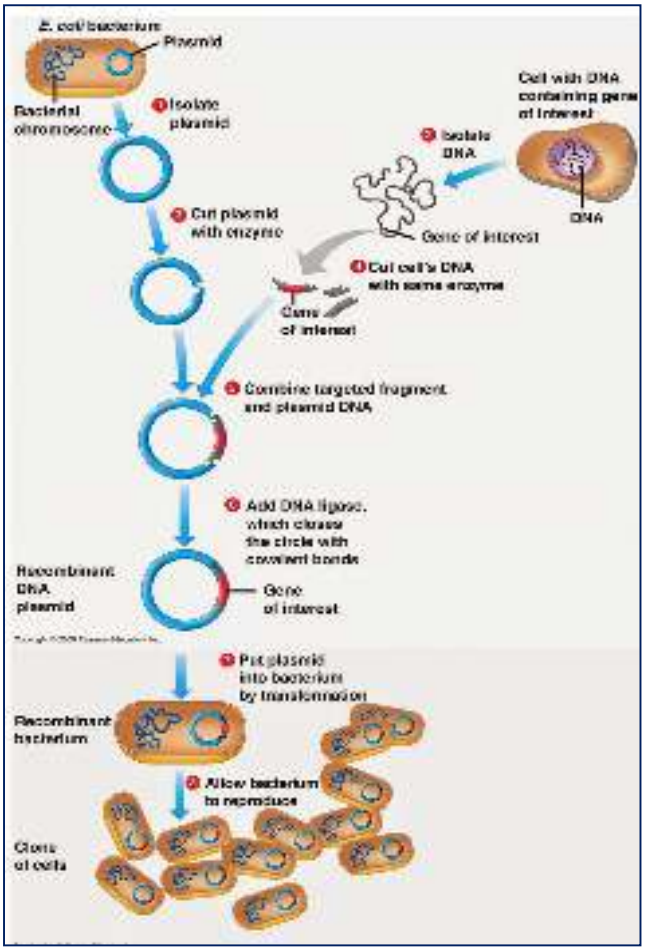


Illustration of Various Applications of r DNA technology


Plasmid vector
DNA fragment to be cloned
Enzymatically insert DNA into plasmid vector
Recombinant plasmid
Culture on nutrient agar plates containing ampicillin
Bacterial chromosome
Transformed E. coli cell survives
Cells that do not take up plasmid die on ampicillin plates
Independent plasmid replication
Cell multiplication
Colony of cells each containing copies of the same recombinant plasmid



Steps in Gene Cloning

The basic 7 steps involved in gene cloning are:

1. Isolation of DNA [gene of interest] fragments to be cloned.
2. Insertion of isolated DNA into a suitable vector to form recombinant DNA.
3. Introduction of recombinant DNA into a suitable organism known as host.
4. Selection of transformed host cells and identification of the clone containing the gene of interest.
5. Multiplication/Expression of the introduced Gene in the host.
6. Isolation of multiple gene copies/Protein expressed by the gene.
7. Purification of the isolated gene copy/protein

1. Humulin
2. Humatrop
3. Activase
4. Monoclonal antibodies by Hybridoma technique
5. Intron a
6. Recombivax HB (Hepatitis B)

1. Insulin vs. Human Insulin Hormone/Humulin

Insulin is a 51 Amino Acid polypeptide weighing 5808 Da, consisting of A and B subunits, connected by two disulphide bridges, with subunit A having an intra-subunit bridge. Proinsulin contains a C subunit which can be proteolytically cleaved by endopeptidases (pro-hormone convertase and carboxy peptidase).

It was first isolated by Frederick G. Banting and Charles H. Best in 1921 at Canada. Human Insulin Hormone (Humulin) is the first product of recombinant DNA technology, first synthesized in 1978 by Genentech.

What is Humulin?

Humulin is synthetic human insulin prepared by using genetic engineering. Humulin is manufactured from DNA sources in laboratory, using recombinant DNA technology. Synthetic insulin is also called genetically engineered insulin. The synthetic insulin (Humulin) is as effective as hormone insulin secreted by human pancreas.

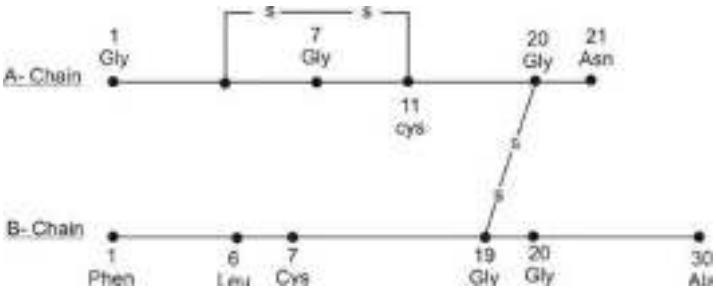
Synthesis of Humulin

In 1978, scientists synthesized human insulin from E.coli bacteria using recombinant DNA technology, by preparing two DNA sequences for A and B chains of human insulin and introduced them in plasmid of E.coli. This led to production of human insulin chain. Eli Lilly, an American company marketed the first human insulin called humulin in 1983. Eli Lilly and Ranbaxy launched a new insulin project namely Humalog (an analog of 5, 6 human insulin), which is more expensive than human insulin products, but have good absorption in body, as compared to other insulin products.

Structure of Insulin

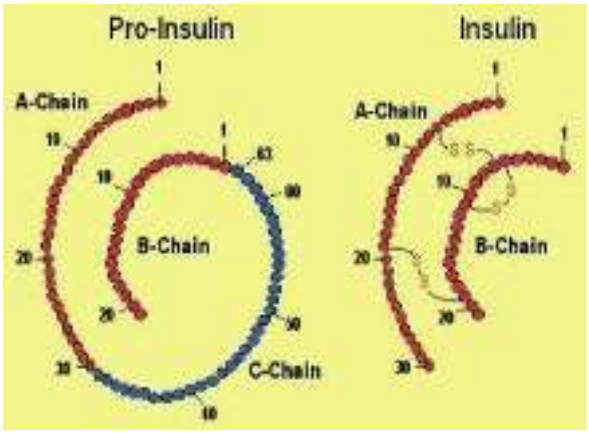
Insulin is a proteinaceous hormone secreted by beta-cells of islets of langerhans of pancreas. Insulin controls, blood sugar level and when there is less secretion of insulin, it results in diabetes (high blood- sugar level).

In 1954, Frederick Sanger determined primary structure of Insulin. Insulin is a protein formed by two polypeptide chains: A-chain and B-chain, interlinked by two sulphide bonds. A-chain is formed of 21 amino acid residue, while B – chain is formed of 30 amino acid residue. The A-chain has N-terminal glycine (GLY) and a C-terminal Asparagine (Asn), while the B-chain has an N-terminal phenylalanine (Phe) and a C terminal Alanine (Ala). Two disulphide bonds (-S-S-) present between two chains lie between cysteine amino acids located at 7th and 20th position of A-chain and 7th and 19th position of B-chain. A third disulfide bond also occurs in the A-chain between cysteine (Cys) amino acids at 6th and 11th position.

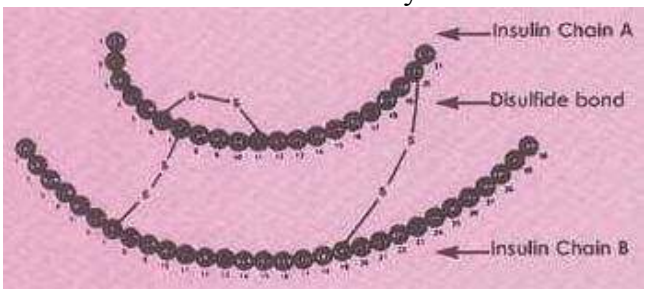


Primary structure of human insulin

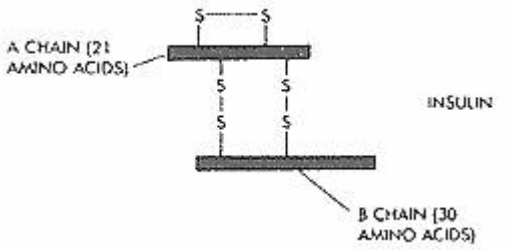
Both chains of insulin are biosynthesized as a single polypeptide chain called proinsulin (inactive insulin), in which A and B chains are interlinked by a connecting polypeptide of 33 amino acids.



Proinsulin where A-chain and B-chain connected by C-chain and insulin without C-chain



Active Insulin, Chance, R. and Frank B. – Research, development, production and safety of Biosynthetic Human Insulin.



1. Synthesis of gene (DNA) for human insulin artificially
2. Culturing recombinant E.coli in bioreactors
3. Purification of Humulin
4. Insertion of human insulin gene into plasmid
5. Introduction of recombinant plasmid into E.coli
6. Extraction of recombinant gene product from E.coli

Advantages of Humulin over Insulin

Earlier insulin required for diabetes was extracted from pancreas of slaughtered cattle, pigs or salmon. The process was quite tedious and difficult and yields of insulin would be low. This extracted insulin in some patients, developed allergy or other side effects due to foreign protein. Due to disadvantages of animal insulin and advantages of humulin, humulin is regarded superior to animal insulin Humulin is considered better than animal insulin because:

- Humulin is absorbed more rapidly and show its effectiveness in short duration.
- Humulin causes fewer allergic and autoimmune reactions as compared to animal insulin.
- Humulin is less expensive than animal insulin
- It is due to above advantages, now almost all insulin marketed is human insulin.

Disadvantages of Humulin over Insulin

Now most of the diabetic patients are treated with synthetic human insulin. Small group of patients claim hat episodes of hyperglycaemic complications have been increased after shifting from animal origin insulin to humulin. No study till date shows the difference between the frequency of hyperglycaemic complications in patient using humulin and animal origin insulin.

2. Humatrop (Human Growth Hormone) production

Humatrope is a man-made form of human growth hormone. It was first approved in 1987 to treat children who are growing slowly because they do not make enough growth hormone on their own. Humatrope is available in 6 mg, 12 mg, and 24 mg cartridges for use in a HumatroPen® injection device.

Growth hormone is produced by the pituitary gland. It regulates the growth and development. Growth hormone stimulates overall body growth by increasing the cellular uptake of amino acids, and protein synthesis, and promoting the use of fat as body fuel.
Insufficient human growth hormone (hGH) in young children results in retarded growth, clinically referred to as pituitary dwarfism. The child usually is less than four feet in height, and has chubby face and abundant fat around the waist.

Traditional treatment for dwarfism:

The children of pituitary dwarfism were treated with regular injections of growth hormone extracted from the brains of deceased humans. It may be noted that only human growth hormone is effective for treatment of dwarfism. (This is in contrast to diabetes where animal insulin's are employed).

Production of recombinant hGH:

Biotechnologists can now produce hGH by genetic engineering. The technique adopted is quite comparable with that of insulin production. The procedure essentially consists of inserting hGH gene into E. coli plasmid, culturing the cells and isolation of the hGH from the extracellular medium.

Limitation in hGH production:

The hGH is a protein comprised of 191 amino acids. During the course of its natural synthesis in the body, hGH is tagged with a single peptide (with 26 amino acids). The signal

peptide is removed during secretion to release the active hGH for biological functions. The entire process of hGH synthesis goes on in an orderly fashion in the body.

However, signal peptide interrupts hGH production by recombinant technology. The complementary DNA (cDNA) synthesized from the mRNA encoding hGH is inserted into the plasmid. The plasmid containing E. coli when cultured, produces full length hGH along with signal peptide. But E. coli cannot remove the signal peptide.

Further, it is also quite difficult to get rid of signal peptide by various other means. Theoretically, cDNA encoding signal peptide can be cut to solve these problems. Unfortunately, there is no restriction endonuclease to do this job, hence this is not possible.

A novel approach for hGH production:

Biotechnologists have resolved the problem of signal peptide interruption by a novel approach (Fig. 15.2). The base sequence in cDNA encoding signal peptide (26 amino acids) plus the neighbouring 24 amino acids (i.e a. total 50 amino acids) is cut by restriction endonuclease *E CoRI.*

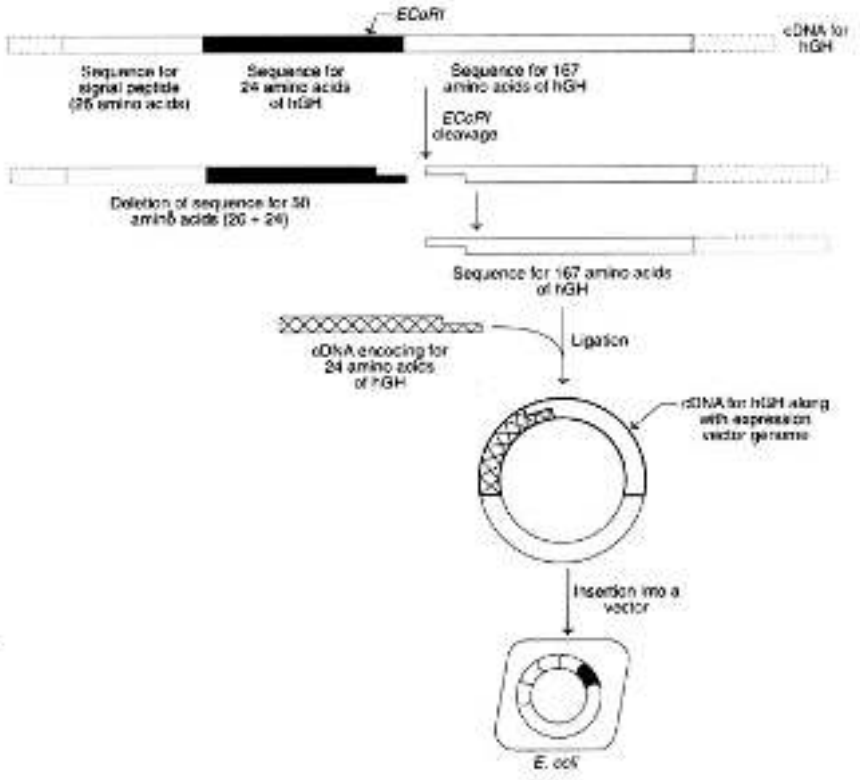


Fig. 15.2 : The production of recombinant human growth hormone (cDNA–Complementary DNA, hGH–Human growth hormone)

3. Alteplase (Rx) - Activase -Tissue Plasminogen Activator

Tissue Plasminogen Activator (tPA) is a naturally occurring protease enzyme that helps to dissolve blood clots. tPA is a boon for patients suffering from thrombosis. The majority of natural deaths worldwide are due to a blockade of cerebral or coronary artery by a blood clot, technically called as thrombus. The phenomenon of thrombus blockage of blood vessels is referred to as thrombosis.

Chemically, thrombus consists of a network of fibrin, formed from the fibrinogen. In the normal circumstances, plasmin degrades fibrin and dissolves blood clots. This plasmin is actually produced by activation of plasminogen by tissue plasminogen activator (Fig. 15.3)

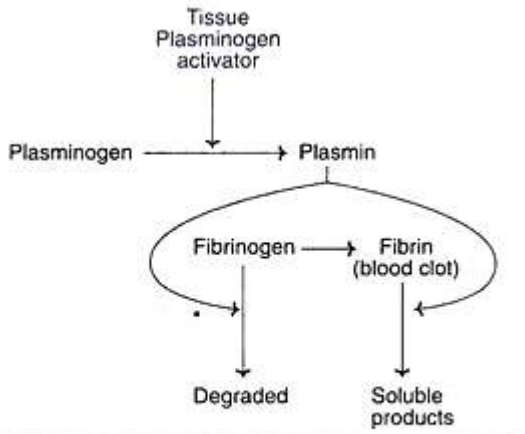


Fig. 15.3 : Role of tissue plasminogen activator in dissolving blood clots.

The natural biological systems is however, not that efficient to remove the blood clots through this machinery. Tissue plasminogen activator is very useful as a therapeutic agent in dissolving blood clots (thrombi) by activating plasminogen. By removing the arterial, thrombi, the possible damage caused by them on heart and brain could be reduced.

Production of recombinant tPA:

DNA technologists synthesized the complementary DNA (cDNA) molecule for tissue plasminogen activator. This cDNA was then attached to a synthetic plasmid and introduced into mammalian cells (Fig. 15.4). They were cultured and tPA-producing cells were selected by using methotrexate to the medium.

tPA-producing cells were transferred to an industrial tank (fermenter). tPA, secreted into the culture medium, is isolated for therapeutic purpose. It may be noted here that tPA was the first pharmaceutical product to be produced by mammalian cell culture.

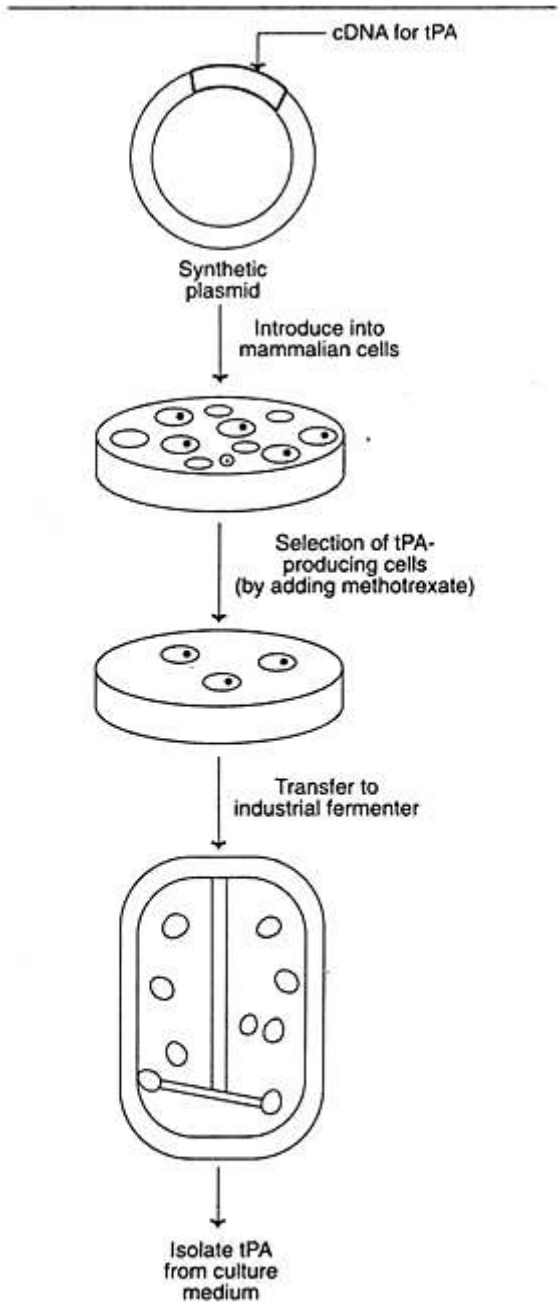


Fig. 15.4 : The production of recombinant tissue plasminogen activator (tPA).

Recombinant tPA has been in use since 1987 for treatment of patients with acute myocardial infarction or stroke. Gene-tech was the first to market tPA with a trade name ***Activase.***

Activase is used to dissolve blood clots that have formed in the blood vessels. It is used immediately after symptoms of a heart attack occur to improve patient survival. It is also used after symptoms of a stroke and to treat blood clots in the lungs (pulmonary embolism).

4. Hybridoma technology - Monoclonal antibodies (mAbs)

What is Hybridoma technology?

Hybridoma technology is a well-established method to produce monoclonal antibodies (mAbs) specific to antigens of interest. Hybridoma cell lines are formed via fusion between a short-lived antibody-producing B cell and an immortal myeloma cell.

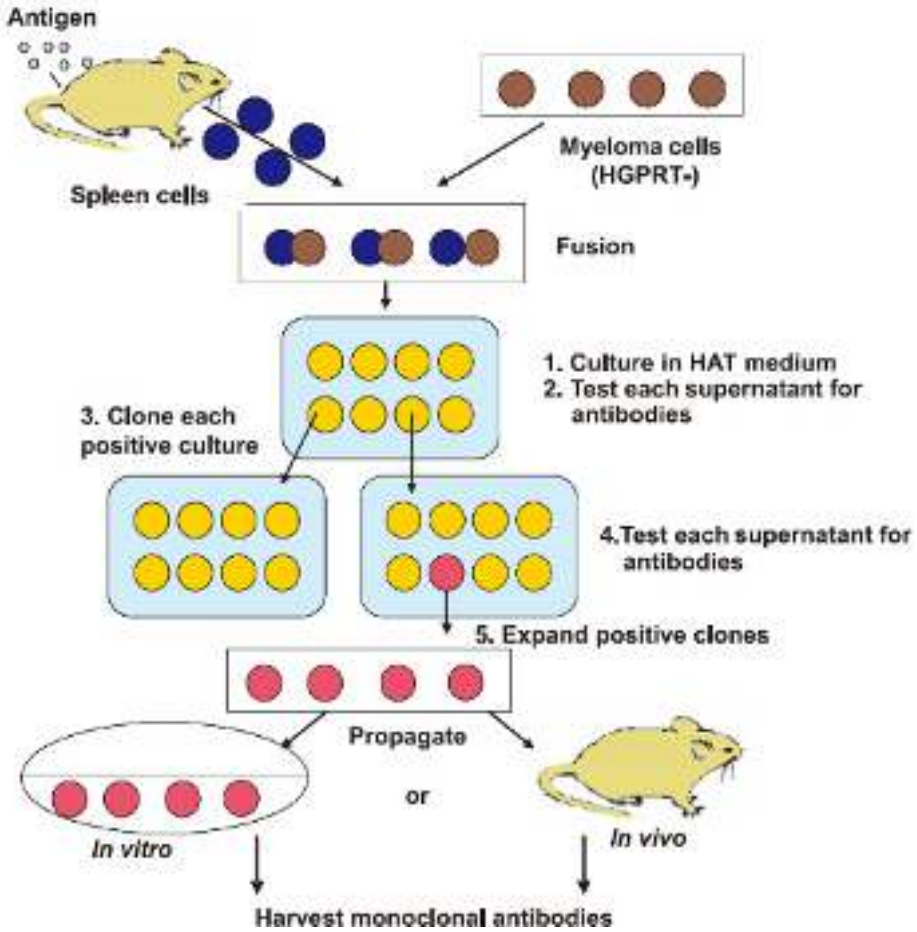
Hybridoma technology was discovered in 1975 by two scientists, Georges Kohler and Cesar Milstein. They wanted to create immortal hybrid cells by fusing normal B cells from immunized mice with their myeloma cells.

Their discovery is considered one of the greatest breakthroughs in the field of biotechnology. For the past decades, hybridomas have fuelled the discovery and production of antibodies for a multitude of applications.

Steps Involved in Hybridoma Technology

Hybridoma technology is composed of several technical procedures, including antigen preparation, animal immunization, cell fusion, hybridoma screening and sub cloning, as well as characterization and production of specific antibodies.

mAb generation by the hybridoma approach requires knowledge of multiple disciplines and practice of versatile technical skills, ranging from animal handling, immunology to cellular and molecular biology. Generation and identification of high-quality hybridoma clones is a comprehensive and labor-intensive process, and requires months of work during the time frame from immunization to specific hybridoma identification.



1) Cell fusion

Polyethylene glycol (PEG) and electro fusion are commonly used to induce cell fusion in hybridoma production. PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei. This hetero karyon retains these nuclei until the nuclear membranes dissolve before mitosis. Electrofusion joins the membranes of neighbouring cells by the application of a pulsed electrical field. Electrofusion is more efficient than PEG and the results are reproducible.

2) Hybridoma screening

Even in the most efficient hybridoma fusions, only about 1% of the starting cells are fused and only about 1 in 10^5 form viable hybrids. This leaves a large number of unfused cells still in culture. The cells from the immunized animal (antibody secreting cell) do not continue to grow in tissue culture and so do not confuse further work. However, the myeloma cells are well adapted to tissue culture and must be killed, which can be achieved by drug selection.

Commonly, the myeloma cells have a defective HGPRT enzyme (hypoxanthine-guanine phosphoribosyl transferase), blocking their ability to use the salvage pathway. These cells containing a non-functional HGPRT protein will die in HAT medium. Only the hybridoma cells have got the ability to divide and proliferate on the HAT medium because genome from the B-lymphocyte makes them HGPRT positive and genome from the myeloma cells they can divide indefinitely.

3) mAb production

Hybridoma antibodies can be produced in vitro and in vivo.

For production of monoclonal antibodies in vitro, hybridomas are expanded by transfer to 24 well tissue culture plates followed by 25 cm^2 flasks and a 75cm^2 flask containing suitable medium. The cell density is maintained between 10^5 and 10^6 cells/ml. Typical culture supernatants yield up to l00μg/ml of antibody, the exact amount depending upon the cell density and rate of growth. Culture in vitro provides a more pure preparation of antibody. Sino Biological can offer serum-free hybridoma production service by the use of serum-free medium.

For producing monoclonal antibodies in vivo, mice are primed by intra peritoneal injection with 10^5 - 10^7 hybridoma cells. The rate of growth of the resulting ascites tumour is in general very variable and can be from less than two or more than five weeks. The ascites fluid can be collected from an anaesthetized mouse. It is possible to obtain 10 ml of ascites fluid or more from a mouse by regular tapping. Ascites fluid will be contaminated with mouse imunoglobulins to a small extent and if a very pure antibody is required this may prove inconvenient.

Applications of Hybridoma Technology

mAb therapeutics

Compared with other biologics, mAbs are able to maintain an extremely high affinity towards their target. Due to this high affinity and specificity, researchers began investigating the therapeutic potential of mAbs as metabolic activators, inhibitors and immuno-modulators. While the first few US FDA-approved mAb therapeutics, such as muromonab-CD3, were generated solely in mice, it became evident that in order to avoid immune rejection, future

mAb-based therapeutics needed to undergo humanization. Since the approval of muromonab-CD3 in 1986, the FDA has approved approximately 80 more mAb therapeutics for diseases ranging from autoimmune disorders, to inflammatory diseases, HIV and cancer. Interestingly, despite the discovery of combinatorial display libraries in 1984 as an alternative mAb discovery platform, the majority of these mAb therapeutics were originally discovered using hybridoma technology in either fully murine or humanized mice. The reason for this preference is likely attributed to the natural ability of the murine immune system to generate highly specific mAbs that elicit strong constant domain functionality with limited immuno reactivity after humanization.

5. Interferons / Intron a:

Interferon is an antiviral substance, and is the first line of defense against viral attacks. The term interferon has originated from the interference of this molecule on virus replication. It was originally discovered in 1957 by Alick Isaacs and Jean Lindemann and was considered to be a single substance.

It is now known that interferon actually consists of a group of more than twenty substances with molecular weights between 20,000-30,000daltons. All the interferons are proteins in nature and many of them are glycoproteins. They are broadly categorized into three groups based on their structure and function,

- ✓ Interferon-α (IFN-α)
- ✓ Interferon-β (IFN-β)
- ✓ Interferon-γ (IFN-γ)

Interferon's (IFNs) are proteins made and released by host cells in response to the presence of pathogens such as viruses, bacteria, parasites or tumour cells. They allow for communication between cells to trigger the protective defences of the immune system that eradicate pathogens or tumours.

IFNs belong to the large class of glycoproteins known as cytokines. Interferons are named after their ability to "interfere" with viral replication within host cells.

IFNs have other functions: they activate immune cells, such as natural killer cells and macrophages; they increase recognition of infection or tumour cells by up-regulating antigen presentation to T lymphocytes; and they increase the ability of uninfected host cells to resist new infection by virus. Certain symptoms, such as aching muscles and fever, are related to the production of IFNs during infection.

About ten distinct IFNs have been identified in mammals; seven of these have been described for humans. They are typically divided among three IFN classes: Type I IFN, Type II IFN, and Type III IFN. IFNs belonging to all IFN classes are very important for fighting viral infections. Interferon type I: All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains. The type I interferons present in humans are IFN-α, IFN-β and IFN-ω.

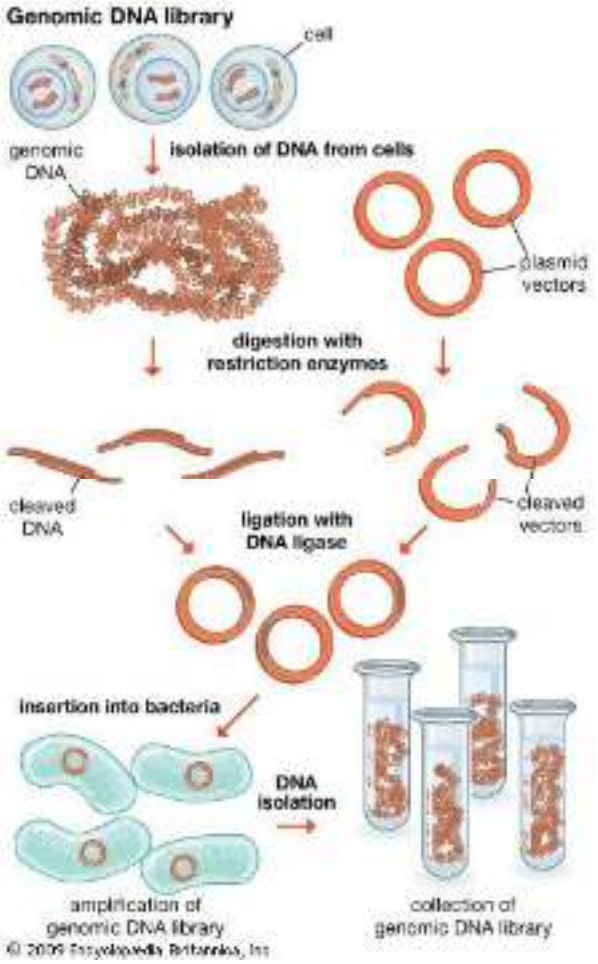
Isolation of interferons in the early years:

Blood was the only source of interferons earlier. The procedure was very tedious and the quantity of interferons isolated was very little. Thus, as much as 50,000 litres of human blood was required to get just 100 mg of interferons. Therefore, it was very difficult to conduct research or use interferons for therapeutic purposes.

Now it is possible to produce interferons by r DNA technology at much cheaper rate.

Production of recombinant interferons:

The complementary DNA (cDNA) was synthesized from the mRNA of a specific interferon. This is inserted to a vector (say plasmid) which is introduced into *E. coli* or other cells. The interferon can be isolated from the culture medium. This is the basic mechanism of producing recombinant interferons. The production of interferons is relatively less in bacterial hosts, although *E. coli* was the first to be used. This is mainly because most interferons are glycoproteins in nature and bacteria do not possess the machinery for glycosylation of proteins.



6. Recombivax HB (Hepatitis B)

A vaccine is a biological preparation that improves immunity to a particular disease. Injection of a killed microbe in order to stimulate the immune system against the microbe, thereby preventing disease.

Vaccine types:

- Live, attenuated vaccines
- Inactivated vaccine
- Subunit vaccine
- Toxoid vaccine
- Conjugated vaccine
- DNA vaccine
- **Recombinant vector vaccines**

Recombinant vector vaccines

Vaccine antigens may also be produced by genetic engineering technology. These products are sometimes referred to as recombinant vaccines

- **Subunit vaccines:**

These are the components of the pathogenic organisms. Subunit vaccines include protein, peptides and DNA.

- **Attenuated recombinant vaccines:**

These are the genetically modified pathogenic organisms that are made non-pathogenic and used as vaccines.

- **Vector recombinant vaccines:**

These are the genetically modified viral vectors that can be used as vaccines against certain pathogens.

Hepatitis B

Hepatitis B is a widespread disease in man. It primarily affects liver causing chronic hepatitis, cirrhosis and liver.

The gene encoding for hepatitis B surface antigen (HBs Ag) has been identified. The HBs Ag vaccine as a subunit vaccine is produced by cloning HBs Ag gene in yeast cells (*Sacchromyces cerevisiae*).

General features of nucleic acid of Hepatitis B Virus

HB virus has been identified as a 42-nm particle containing a double stranded circular DNA molecule of about 3Kb size. DNA genome has a relative molecular mass of approximately 2 X 10.

Plasma of human has been detected to have varying amount of HB antigens. Three types of viral coat proteins are recognized to be antigenic

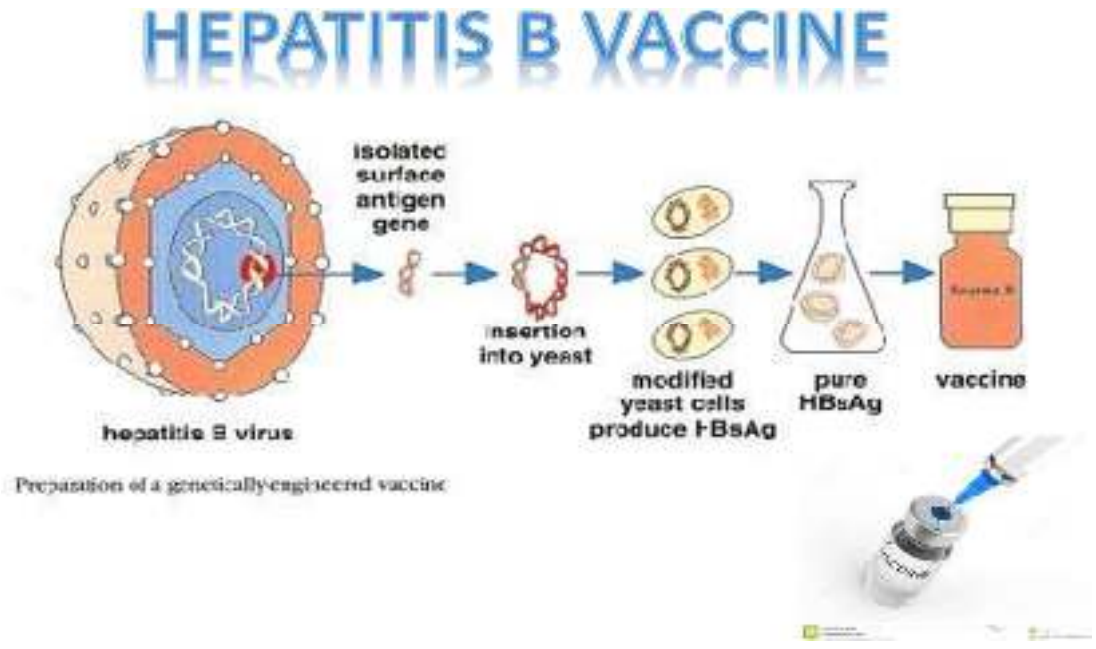
- viral surface antigen (HBs Ag)
- viral core antigen (HBc Ag)
- the e-antigen (HBe Ag)

Surface antigen HBs AG is found as 18-22 nm spherical or tubular form particles. Recently HBs Ag gene or it's subunits are used for the production of recombinant Hepatitis B vaccine.

General steps for Recombinant Hepatitis B Vaccine production

Production of these genes is needed in order to get production of vaccines on a large scale. A general procedure for the production of recombinant Hepatitis B vaccines are described here

1. HBs antigen producing gene is isolated from the HB virus by normal isolation process (cell lysis, protein denaturation, precipitation, centrifugation and drying).
2. A plasmid DNA is extracted from a bacterium- E.coli and is cut with restriction enzyme- Eco RI forming the plasmid vector
3. The isolated HBs antigen producing gene is located and inserted into the bacterial plasmid vector on forming the recombinant DNA.
4. This recombinant DNA, containing the target gene, is introduced into a yeast cell forming the recombinant yeast cell.
5. The recombinant yeast cell multiplies in the fermentation tank and produces the HBs antigens.
6. After 48 hours, yeast cells are ruptured to free HBs Ag. The mixture is processed for extraction.
7. The HBs antigens are purified.
8. HBs Ag are combined with preserving agent and other ingredients and bottled. Now it is ready for vaccination in humans.



Preparation of a genetically-engineered vaccine

UNIT II - R DNA TECHNOLOGY

Introduction

Genetic engineering is a tool of biotechnology sophisticated and most advanced. Genetic engineering comprises multiple techniques for the **intentional manipulation** of genetic material (primarily deoxyribonucleic acid, or DNA) to alter, repair, or enhance form or function.

Genetic engineering, also called recombinant DNA technology, involves the group of techniques used to cut up and join together genetic material, especially DNA from different biological species, and to introduce the resulting hybrid DNA into an organism in order to form new combinations of heritable genetic material.

- Recombinant **DNA** technology refers to the joining together of DNA molecules from two different species that are inserted into a host organism to produce new genetic combinations that are of value to science, medicine, agriculture, and industry.
- Recombinant DNA (rDNA), on the other hand is the general name for a piece of DNA that has been created by the combination of at least two strands.
- They are DNA molecules formed by laboratory methods of genetic recombination (such as molecular cloning) to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in the genome.

Steps of Genetic Recombination Technology

1. Isolation of Genetic Material

- The first step in rDNA technology is to isolate the desired DNA in its pure form i.e. free from other macromolecules.
- Since DNA exists within the cell membrane along with other macromolecules such as RNA, polysaccharides, proteins, and lipids, it must be separated and purified which involves enzymes such as lysozymes, cellulase, chitinase, ribonuclease, proteases etc.
- Other macromolecules are removable with other enzymes or treatments. Ultimately, the addition of ethanol causes the DNA to precipitate out as fine threads. This is then spooled out to give purified DNA.

2. Restriction Enzyme Digestion

- ✓ Restriction enzymes act as **molecular scissors** that cut DNA at specific locations. These reactions are called **'restriction enzyme digestions'**.
- ✓ They involve the incubation of the purified DNA with the selected restriction enzyme, at conditions optimal for that specific enzyme.
- ✓ The technique '**Agarose Gel Electrophoresis'** reveals the progress of the restriction enzyme digestion.
- ✓ This technique involves running out the DNA on an agarose gel. On the application of current, the negatively charged DNA travels to the positive electrode and is separated out based on size. This allows separating and cutting out the digested DNA fragments.
- ✓ The vector DNA is also processed using the same procedure.

3. Amplification Using PCR

- ✓ Polymerase Chain Reaction or PCR is a method of making multiple copies of a DNA sequence using the enzyme – DNA polymerase in vitro.
- ✓ It helps to amplify a single copy or a few copies of DNA into thousands to millions of copies.
- ✓ PCR reactions are run on 'thermal cyclers' using the following components:
- ✓ Template – DNA to be amplified
- ✓ Primers – small, chemically synthesized oligonucleotides that are complementary to a region of the DNA.
- ✓ Enzyme – DNA polymerase
- ✓ Nucleotides – needed to extend the primers by the enzyme.
- ✓ The cut fragments of DNA can be amplified using PCR and then ligated with the cut vector.

4. Ligation of DNA Molecules

- ✓ The purified DNA and the vector of interest are cut with the same restriction enzyme.
- ✓ This gives us the cut fragment of DNA and the cut vector, that is now open.
- ✓ The process of joining these two pieces together using the enzyme 'DNA ligase' is 'ligation'.
- ✓ The resulting DNA molecule is a hybrid of two DNA molecules – the interest molecule and the vector. In the terminology of genetics this intermixing of different DNA strands is called recombination.
- ✓ Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and the technology is referred to as the recombinant DNA technology.

5. Insertion of Recombinant DNA Into Host

- ✓ In this step, the recombinant DNA is introduced into a recipient host cell mostly, a bacterial cell. This process is '***Transformation'.***
- ✓ Bacterial cells do not accept foreign DNA easily. Therefore, they are treated to make them 'competent' to accept new DNA. The processes used may be thermal shock, Ca^{++}ion treatment, electroporation etc.

6. Isolation of Recombinant Cells

- ✓ The transformation process generates a mixed population of transformed and non-trans- formed host cells.
- ✓ The selection process involves filtering the transformed host cells only.
- ✓ For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector is employed.
- ✓ For examples, PBR322 plasmid vector contains different marker gene (Ampicillin resistant gene and Tetracycline resistant gene. When pst1 RE is used it knock out Ampicillin resistant gene from the plasmid, so that the recombinant cell become sensitive to Ampicillin.

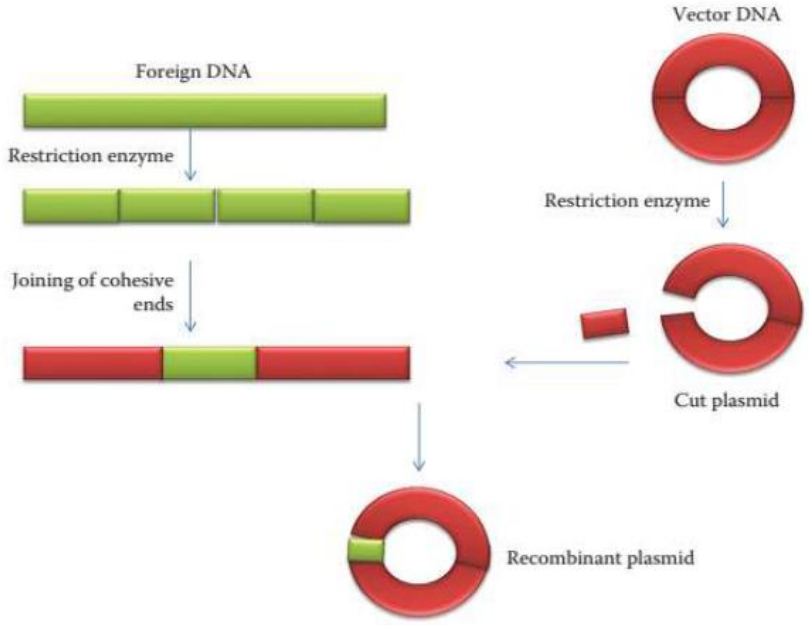


Figure 1: Making of recombinant DNA

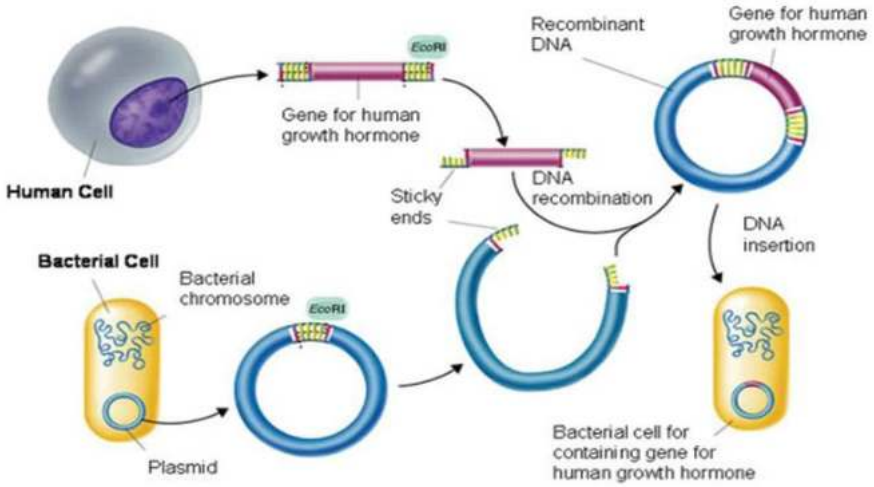


Figure 2: Process of recombinant DNA

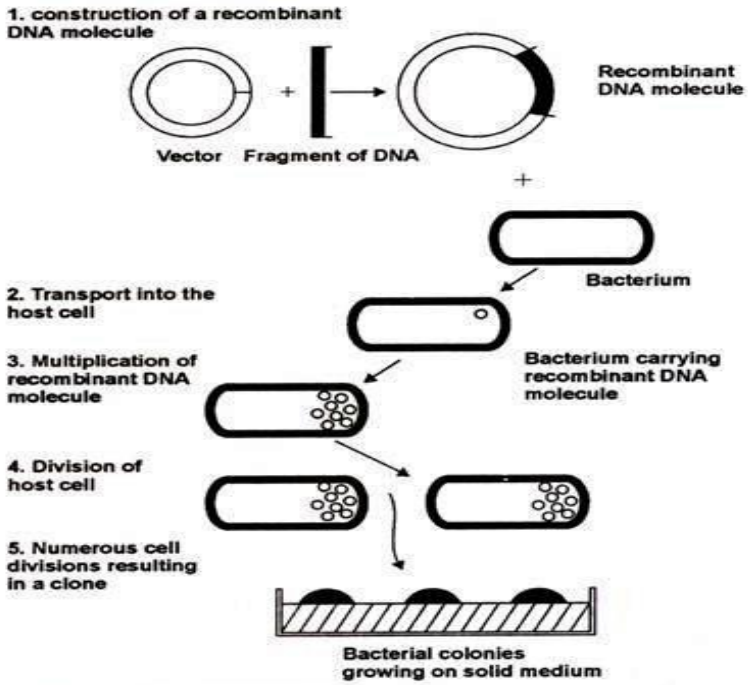


Fig. 1. The basic steps of rec DNA Technology using the bacterial plasmid as cloning vector.

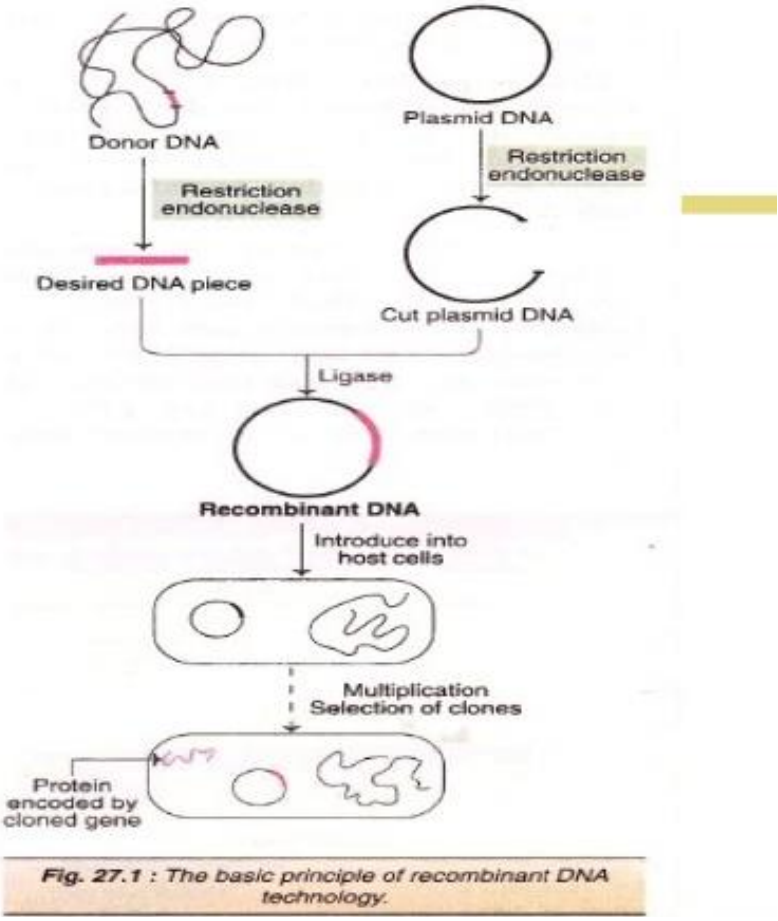


Fig. 27.1 : The basic principle of recombinant DNA technology.

STEPS in r DNA technology

i. Selection and isolation of DNA insert
ii. Selection of suitable cloning vector
iii. Introduction of DNA-insert into vector to form r DNA molecule
iv. r DNA molecule is introduced into a suitable host.
v. Selection of transformed host cells.
vi. Expression and multiplication of DNA-insert in the host.

(i) Selection and isolation of DNA insert:

First step in r DNA technology is the selection of a DNA segment of interest which is to be cloned. This desired DNA segment is then isolated enzymatically. This DNA segment of interest is termed as DNA insert or foreign DNA or target DNA or cloned DNA.

(ii) Selection of suitable cloning vector:

A cloning vector is a self-replicating DNA molecule, into which the DNA insert is to be integrated. A suitable cloning vector is selected in the next step of r DNA technology. Most commonly used vectors are plasmids and bacteriophages.

(iii) Introduction of DNA-insert into vector to form r DNA molecule:

The target DNA or the DNA insert which has been extracted and cleaved enzymatically by the selective restriction endonuclease enzymes [in step (i)] are now ligated (joined) by the enzyme ligase to vector DNA to form a r DNA molecule which is often called as cloning-vector-insert DNA construct.

(iv) r DNA molecule is introduced into a suitable host:

Suitable host cells are selected and the r DNA molecule so formed [in step (iii)] is introduced into these host cells. This process of entry of r DNA into the host cell is called *transformation.* Usually selected hosts are bacterial cells like *E. coli,* however yeast, fungi may also be utilized.

(v) Selection of transformed host cells:

Transformed cells (or recombinant cells) are those host cells which have taken up the r DNA molecule. In this step the transformed cells are separated from the non-transformed cells by using various methods making use of marker gene.

(vi) Expression and Multiplication of DNA insert in the host:

Finally, it is to be ensured that the foreign DNA inserted into the vector DNA is expressing the desired character in the host cells. Also, the transformed host cells are multiplied to obtain sufficient number of copies. If needed, such genes may also be transferred and expressed into another organism.

Tools for Recombinant DNA Technology:

r DNA technology utilizes a number of biological tools to achieve its objectives, most important of them being the enzymes.

Important biological tools for r DNA technology are:

(A) Enzymes:
- a. Restriction Endonucleases
- b. Exonucleases
- c. DNA ligases
- d. DNA polymerase

(B) Cloning Vector

(C) Host organism

(D) DNA insert or foreign DNA

(E) Linker and adaptor sequences.

An account of all these biological tools of genetic engineering is given below:

(A) ENZYMES:

A number of specific enzymes are utilized to achieve the objectives of r DNA technology.

The enzymology of genetic engineering includes the following types of enzymes:

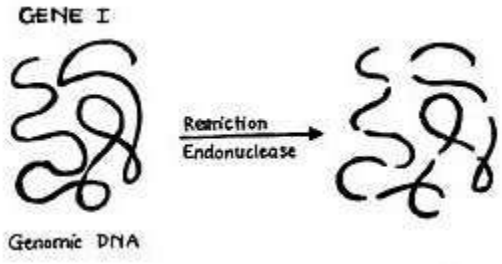
(a) Restriction Endonuclease:

These enzymes serve as important tools to cut DNA molecules at specific sites, which is the basic need for r DNA technology.

These are the enzymes that produce internal cuts (cleavage) in the strands of DNA, only within or near some specific sites called recognition sites/recognition sequences/ restriction sites 01 target sites. Such recognition sequences are specific for each restriction enzyme. Restriction endonuclease enzymes are the first necessity for r DNA technology.

The ability to join DNA molecules together for cloning is dependent on the type of enzymes occurs in bacteria. These restriction enzymes are called restriction endonuclease or molecular scissors.

Restriction endonucleases are of bacterial origin and protect host cells as a weapon against foreign genes breaking them into smaller pieces and destroy them.



DNA converting into fragments of DNA

Types of Restriction Endonucleases:

There are 3 main categories of restriction endonuclease enzymes:

Type-I Restriction Endonucleases-

These are the complex type of endonucleases which cleave only one strand of DNA. These enzymes have the recognition sequences of about 15 bp length. This recognises a specific sequence but cuts the DNA outside the sequence 1000 base pairs away. They are most complex and not useful in gene manipulation as their cleavage sites are non specific. These enzymes with their subunits are responsible for methylation, endonucleolytic activity and site specificity.

Type-II Restriction Endonucleases-

These enzymes show cleavage in both the strands of DNA, are most important endonucleases for gene cloning. They show cleavage only at specific sites and therefore they produce the DNA fragments of a defined length. They cuts DNA within the recognisition sequence and are used for gene manipulation studies.

Type-III Restriction Endonucleases-

These are not used for gene cloning. They are the intermediate enzymes between Type-I and Type-II restriction endonuclease. They cuts the DNA approximately 25 base pairs away from recognisition sites.

Nature of cleavage by Restriction Endonucleases:

They cut the DNA molecule in two ways:

i. Many restriction endonucleases cleave both strands of DNA simply at the same point within the recognition sequence. As a result of this type of cleavage, the DNA fragments with blunt ends are generated.

ii. In the other style of cleavage by the restriction endonucleases, the two strands of DNA are cut at two different points. Such cuts are termed as staggered cuts and this results into the generation of protruding ends i.e., one strand of the double helix extends a few bases beyond the other strand. Such ends are, called cohesive or sticky ends.

(b) Exonucleases:

Exonuclease is an enzyme that removes nucleotides from the ends of a nucleic acid molecule. An exonuclease removes nucleotide from the 5′ or 3′ end of a DNA molecule. An exonuclease never produces internal cuts in DNA.

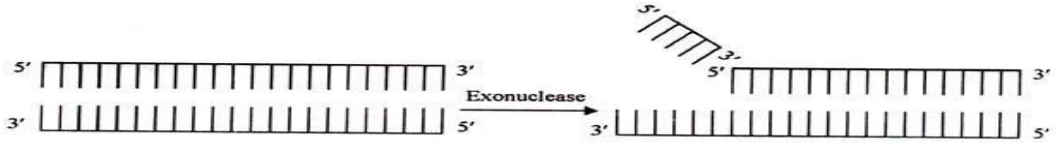
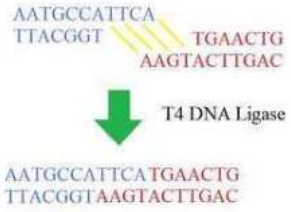


Fig. 4. An Exonuclease activity : Nucleotides are removed from the end of DNA

In r DNA technology, various types of exonucleases are employed like Exonuclease Bal 31, E. coli exonuclease III, Lambda exonuclease, etc.

(c) DNA ligase:

The function of these enzymes is to join two fragments of DNA by synthesizing the phosphodiester bond. They function to repair the single stranded nicks in DNA double helix and in r DNA technology they are employed for sealing the nicks between adjacent nucleotides. This enzyme is also termed as **molecular glue.**



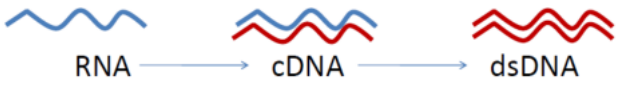
(d) DNA polymerases:

These are the enzymes which synthesize a new complementary DNA strand of an existing DNA or RNA template. A few important types of DNA polymerases are used routinely in genetic engineering. One such enzyme is DNA polymerase ! which, prepared from E coli.

Examples:

DNA polymerase used in genetic engineering is Taq DNA polymerase which is used in PCR (Polymerase Chain Reaction).

Reverse transcriptase is also an important type of DNA polymerase enzyme for genetic engineering. It uses RNA as a template for synthesizing a new DNA strand called as c DNA a e complementary DNA). Its main use is in the formation of c DNA libraries.



Source and cleavage sites of restriction enzymes

Enzyme	Source	*Recognisition sequence*	*Cleavage site*
Hind III	*Haemophilus influenza*	5'AAGCTT 3'TTCGAA	5'---A AGCTT---3' 3'---TTCGA A---5'
Eco RI	*Escherichia coli*	5'GAATTC 3'CTTAAG	5'---G AATTC---3' 3'---CTTAA G---5'
BamHI	*Bacillus amyloliquefaciens*	5'GGATCC 3'CCTAGG	5'---G GATCC---3' 3'---CCTAG G---5'
SalI	*Streptomyces albus*	5'GTCGAC 3'CAGCTG	5'---G TCGAC---3' 3'---CAGCT G---5'
TaqI	*Thermus aquaticus*	5'TCGA 3'AGCT	5'---T CGA---3' 3'---AGC T---5'
Sau3AI	*Staphylococcus aureus*	5'GATC 3'CTAG	5'--- GATC---3' 3'---CTAG ---5'

(B) Cloning Vectors:

It is another important natural tool which geneticists use in r DNA technology. The cloning vector is the DNA molecule capable of replication in a host organism, into which the target DNA is introduced producing the r DNA molecule.

The cloning vector which has only a single site for cutting by a particular restriction endonuclease is considered as a good cloning vector. Different types of DNA molecules may be used as cloning vehicles such as they may be *plasmids, bacteriophages, cosmids, phasmids or artificial chromosomes.*

Features of Cloning Vectors

The cloning vectors possess the following features:

1. A cloning vector should possess an origin of replication so that it can self-replicate inside the host cell.
2. It should have a restriction site for the insertion of the target DNA.
3. It should have a selectable marker with an antibiotic resistance gene that facilitates screening of the recombinant organism.
4. It should be small in size so that it can easily integrate into the host cell.
5. It should be capable of inserting a large segment of DNA.
6. It should possess multiple cloning sites.
7. It should be capable of working under the prokaryotic and eukaryotic systems

Types of Cloning Vectors

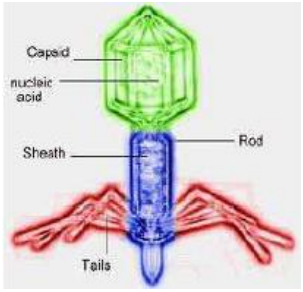
There are the following different types of cloning vectors:

Plasmids

- These were the first vectors used in gene cloning.
- These are found in bacteria, eukaryotes and archaea.

- These are natural, extra chromosomal, self-replicating DNA molecules.
- They have a high copy number and possess antibiotic-resistant genes.
- They encode proteins which are necessary for their own replication.
- *pBR322, pUC18, F-plasmid* are some of the examples of plasmid vectors

Bacteriophage

- These are more efficient than plasmids for cloning large DNA inserts.
- *Phage λ and M13 phage* are commonly used bacteriophages in gene cloning.
- 53 kb DNA can be packaged in the bacteriophage.
- The screening of phage plaques is much easier than the screening of recombinant bacterial colonies.
- 

Phagemids

- These are artificial vectors.
- They are used in combination with M13 phage.
- They possess multiple cloning sites and an inducible lac gene promoter.
- They are identified by blue-white screening.

Bacterial Artificial Chromosomes

- These are similar to *E. coli* plasmids vectors.
- It is obtained from naturally occurring F' plasmid.
- These are used to study genetic disorders.
- They can accommodate large DNA sequences without any risk.

Other cloning vectors include:

- Yeast Artificial Chromosomes
- Cosmids
- Retroviral Vectors
- Human Artificial Chromosomes

(C) Host Organism:

A good host organism is an essential tool for genetic engineering. Most widely used host for r DNA technology is the bacterium *E. coli.* because cloning and isolation of DNA inserts is

very easy in this host. A good host organism is the one which easy to transform and in which the replication of r DNA is easier. There should not be any interfering element against the replication of r DNA in the host cells.

(D) DNA Insert or Foreign DNA:

The desired DNA segment which is to be cloned is called as DNA insert or foreign DNA or target DNA. The selection of a suitable target DNA is the very first step of r DNA technology. The target DNA (gene) may be of viral, plant, animal or bacterial origin.

Following points must be kept in mind while selecting the foreign DNA:

- ✓ It can be easily extracted from source.
- ✓ It can be easily introduced into the vector.
- ✓ The genes should be beneficial for commercial or research point of view.

A number of foreign genes are being cloned for benefit of human beings. Some of these DNA inserts are the genes responsible for the production of insulin, interferon's, lymphotoxins various growth factors, interleukins, etc.

(E) Linker and Adaptor Sequences:

Linkers and adaptors are the DNA molecules which help in the modifications of cut ends of DNA fragments. These can be joined to the cut ends and hence produce modifications as desired. Different types of linkers and adaptors are used for different purposes.

Application of Recombinant DNA technology

r DNA is widely used in biotechnology, medicine & research

- ✓ The most common application of recombinant DNA is in basic research, in which the technology is important to most current work in the biological and biomedical sciences.
- ✓ Recombinant DNA is used to identify, map and sequence genes, and to determine their function.
- ✓ Recombinant proteins are widely used as reagents in laboratory experiments and to generate antibody probes for examining protein synthesis within cells and organisms.
- ✓ Many additional practical applications of recombinant DNA are found in industry, food production, human and veterinary medicine, agriculture, and bioengineering.
- ✓ DNA technology is also used to detect the presence of HIV in a person.
- ✓ Application of recombinant DNA technology in Agriculture – For example, manufacture of Bt-Cotton to protect the plant against ball worms.
- ✓ Application of medicines – Insulin production by DNA recombinant technology is a classic example.
- ✓ Gene Therapy – It is used as an attempt to correct the gene defects which give rise to heredity diseases.
- ✓ Clinical diagnosis – ELISA is an example where the application of recombinant DNA is possible.

Some recombinant DNA products being used in human therapy:

Using procedures like this, many human genes have been cloned in *E. coli* or in yeast. This has made it possible — for the first time — to produce unlimited amounts of human proteins in vitro. Cultured cells (*E. coli*, yeast, mammalian cells) transformed with a human gene are being used to manufacture more than 100 products for human therapy. Some examples:

- Insulin for diabetics
- Factor VIII for males suffering from hemophilia A
- Factor IX for hemophilia B
- Human growth hormone (HGH)
- Erythropoietin (EPO) for treating anemia
- Several types of interferons
- Several interleukins
- Granulocyte-macrophage colony-stimulating factor (GM-CSF) for stimulating the bone marrow after a bone marrow transplant
- Granulocyte colony-stimulating factor (G-CSF) for stimulating neutrophil production (e.g., after chemotherapy) and for mobilizing hematopoietic stem cells from the bone marrow into the blood.
- Tissue plasminogen activator (TPA) for dissolving blood clots
- Adenosine deaminase (ADA) for treating some forms of severe combined immunodeficiency (SCID)
- Parathyroid hormone
- Many monoclonal antibodies
- Hepatitis B surface antigen (HBsAg) to vaccinate against the hepatitis B virus
- C1 inhibitor (C1INH) used to treat hereditary angioedema

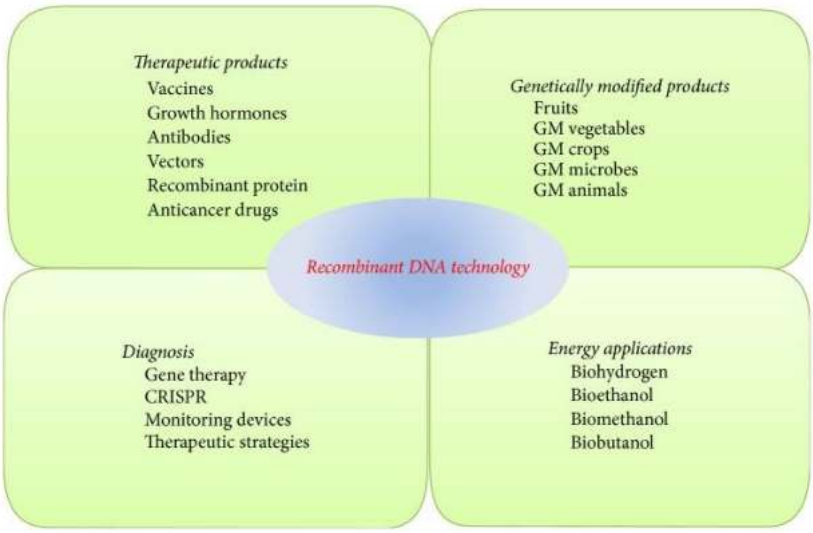
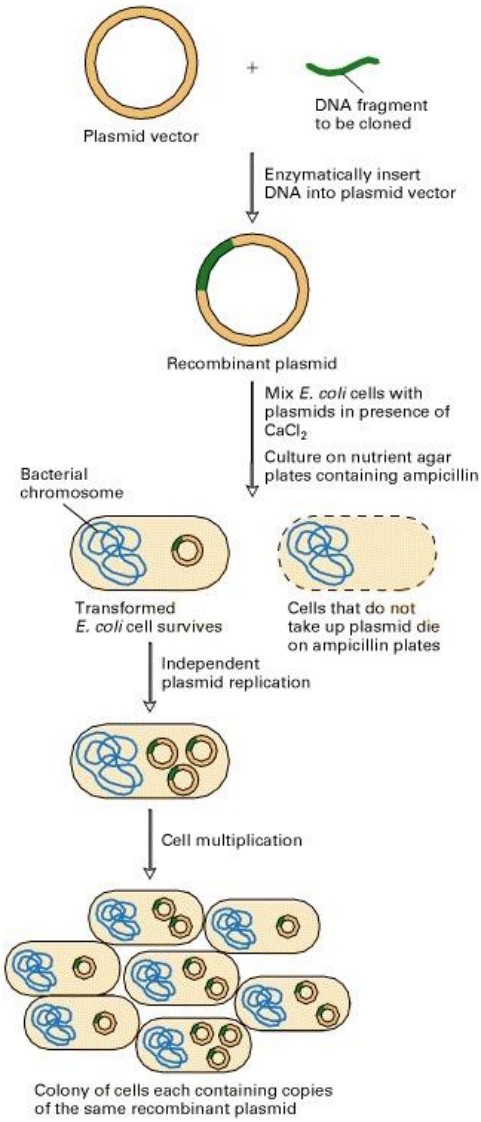
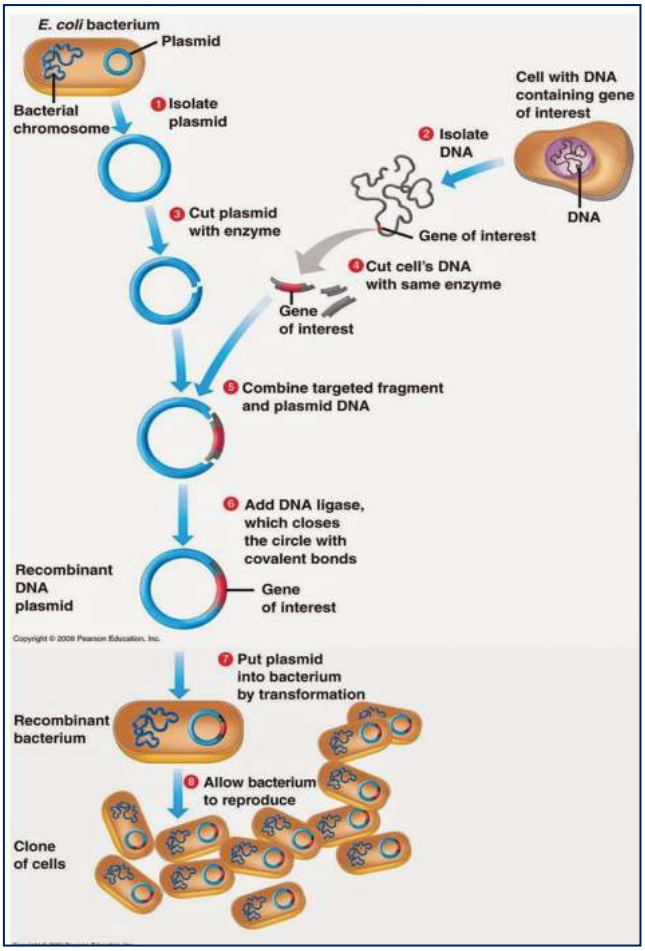


Illustration of Various Applications of r DNA technology


Plasmid vector
+
DNA fragment to be cloned
Enzymatically insert DNA into plasmid vector
Recombinant plasmid
Mix E. coli cells with plasmids in presence of $CaCl_2$
Culture on nutrient agar plates containing ampicillin
Bacterial chromosome
Transformed E. coli cell survives
Cells that do not take up plasmid die on ampicillin plates
Independent plasmid replication
Cell multiplication
Colony of cells each containing copies of the same recombinant plasmid



Steps in Gene Cloning

The basic 7 steps involved in gene cloning are:

1. Isolation of DNA [gene of interest] fragments to be cloned.
2. Insertion of isolated DNA into a suitable vector to form recombinant DNA.
3. Introduction of recombinant DNA into a suitable organism known as host.
4. Selection of transformed host cells and identification of the clone containing the gene of interest.
5. Multiplication/Expression of the introduced Gene in the host.
6. Isolation of multiple gene copies/Protein expressed by the gene.
7. Purification of the isolated gene copy/protein

1. Humulin
2. Humatrop
3. Activase
4. Monoclonal antibodies by Hybridoma technique
5. Intron a
6. Recombivax HB (Hepatitis B)

1. Insulin vs. Human Insulin Hormone/Humulin

Insulin is a 51 Amino Acid polypeptide weighing 5808 Da, consisting of A and B subunits, connected by two disulphide bridges, with subunit A having an intra-subunit bridge. Proinsulin contains a C subunit which can be proteolytically cleaved by endopeptidases (pro-hormone convertase and carboxy peptidase).

It was first isolated by Frederick G. Banting and Charles H. Best in 1921 at Canada. Human Insulin Hormone (Humulin) is the first product of recombinant DNA technology, first synthesized in 1978 by Genentech.

What is Humulin?

Humulin is synthetic human insulin prepared by using genetic engineering. Humulin is manufactured from DNA sources in laboratory, using recombinant DNA technology. Synthetic insulin is also called genetically engineered insulin. The synthetic insulin (Humulin) is as effective as hormone insulin secreted by human pancreas.

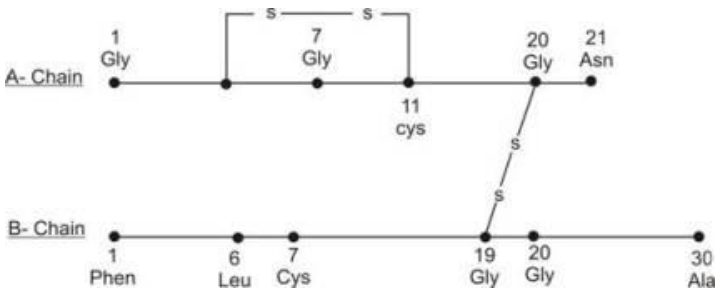
Synthesis of Humulin

In 1978, scientists synthesized human insulin from E.coli bacteria using recombinant DNA technology, by preparing two DNA sequences for A and B chains of human insulin and introduced them in plasmid of E.coli. This led to production of human insulin chain. Eli Lilly, an American company marketed the first human insulin called humulin in 1983. Eli Lilly and Ranbaxy launched a new insulin project namely Humalog (an analog of 5, 6 human insulin), which is more expensive than human insulin products, but have good absorption in body, as compared to other insulin products.

Structure of Insulin

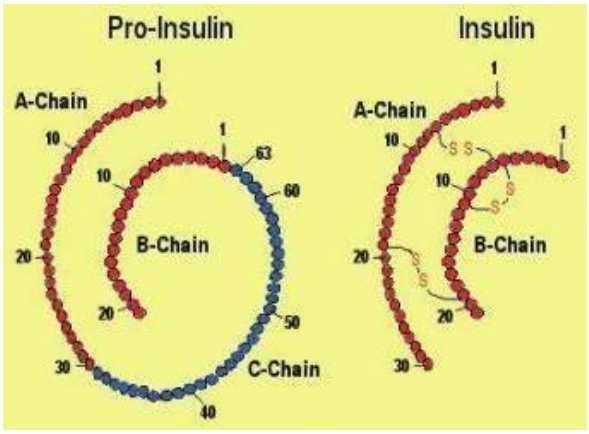
Insulin is a proteinaceous hormone secreted by beta-cells of islets of langerhans of pancreas. Insulin controls, blood sugar level and when there is less secretion of insulin, it results in diabetes (high blood- sugar level).

In 1954, Frederick Sanger determined primary structure of Insulin. Insulin is a protein formed by two polypeptide chains: A-chain and B-chain, interlinked by two sulphide bonds. A-chain is formed of 21 amino acid residue, while B – chain is formed of 30 amino acid residue. The A-chain has N-terminal glycine (GLY) and a C-terminal Asparagine (Asn), while the B-chain has an N-terminal phenylalanine (Phe) and a C terminal Alanine (Ala). Two disulphide bonds (-S-S-) present between two chains lie between cysteine amino acids located at 7th and 20th position of A-chain and 7th and 19th position of B-chain. A third disulfide bond also occurs in the A-chain between cysteine (Cys) amino acids at 6th and 11th position.

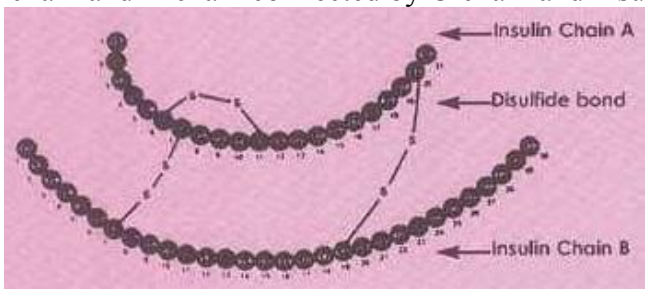


Primary structure of human insulin

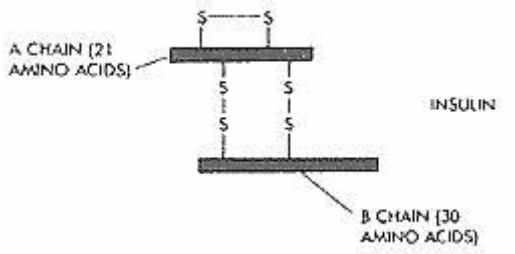
Both chains of insulin are biosynthesized as a single polypeptide chain called proinsulin (inactive insulin), in which A and B chains are interlinked by a connecting polypeptide of 33 amino acids.



Proinsulin where A-chain and B-chain connected by C-chain and insulin without C-chain



Active Insulin, Chance, R. and Frank B. – Research, development, production and safety of Biosynthetic Human Insulin.



1. Synthesis of gene (DNA) for human insulin artificially
2. Culturing recombinant E.coli in bioreactors
3. Purification of Humulin
4. Insertion of human insulin gene into plasmid
5. Introduction of recombinant plasmid into E.coli
6. Extraction of recombinant gene product from E.coli

Advantages of Humulin over Insulin

Earlier insulin required for diabetes was extracted from pancreas of slaughtered cattle, pigs or salmon. The process was quite tedious and difficult and yields of insulin would be low. This extracted insulin in some patients, developed allergy or other side effects due to foreign protein. Due to disadvantages of animal insulin and advantages of humulin, humulin is regarded superior to animal insulin Humulin is considered better than animal insulin because:

- Humulin is absorbed more rapidly and show its effectiveness in short duration.
- Humulin causes fewer allergic and autoimmune reactions as compared to animal insulin.
- Humulin is less expensive than animal insulin
- It is due to above advantages, now almost all insulin marketed is human insulin.

Disadvantages of Humulin over Insulin

Now most of the diabetic patients are treated with synthetic human insulin. Small group of patients claim hat episodes of hyperglycaemic complications have been increased after shifting from animal origin insulin to humulin. No study till date shows the difference between the frequency of hyperglycaemic complications in patient using humulin and animal origin insulin.

2. Humatrop (Human Growth Hormone) production

Humatrope is a man-made form of human growth hormone. It was first approved in 1987 to treat children who are growing slowly because they do not make enough growth hormone on their own. Humatrope is available in 6 mg, 12 mg, and 24 mg cartridges for use in a HumatroPen® injection device.

Growth hormone is produced by the pituitary gland. It regulates the growth and development. Growth hormone stimulates overall body growth by increasing the cellular uptake of amino acids, and protein synthesis, and promoting the use of fat as body fuel.
Insufficient human growth hormone (hGH) in young children results in retarded growth, clinically referred to as pituitary dwarfism. The child usually is less than four feet in height, and has chubby face and abundant fat around the waist.

Traditional treatment for dwarfism:

The children of pituitary dwarfism were treated with regular injections of growth hormone extracted from the brains of deceased humans. It may be noted that only human growth hormone is effective for treatment of dwarfism. (This is in contrast to diabetes where animal insulin's are employed).

Production of recombinant hGH:

Biotechnologists can now produce hGH by genetic engineering. The technique adopted is quite comparable with that of insulin production. The procedure essentially consists of inserting hGH gene into E. coli plasmid, culturing the cells and isolation of the hGH from the extracellular medium.

Limitation in hGH production:

The hGH is a protein comprised of 191 amino acids. During the course of its natural synthesis in the body, hGH is tagged with a single peptide (with 26 amino acids). The signal

peptide is removed during secretion to release the active hGH for biological functions. The entire process of hGH synthesis goes on in an orderly fashion in the body.

However, signal peptide interrupts hGH production by recombinant technology. The complementary DNA (cDNA) synthesized from the mRNA encoding hGH is inserted into the plasmid. The plasmid containing E. coli when cultured, produces full length hGH along with signal peptide. But E. coli cannot remove the signal peptide.

Further, it is also quite difficult to get rid of signal peptide by various other means. Theoretically, cDNA encoding signal peptide can be cut to solve these problems. Unfortunately, there is no restriction endonuclease to do this job, hence this is not possible.

A novel approach for hGH production:

Biotechnologists have resolved the problem of signal peptide interruption by a novel approach (Fig. 15.2). The base sequence in cDNA encoding signal peptide (26 amino acids) plus the neighbouring 24 amino acids (i.e a. total 50 amino acids) is cut by restriction endonuclease *E CoRI.*

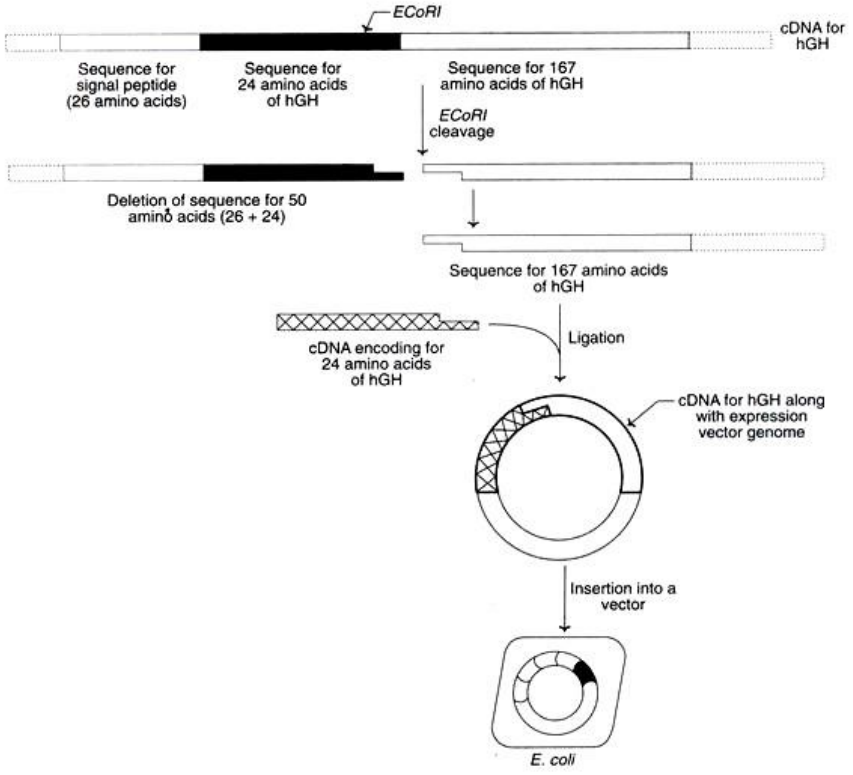


Fig. 15.2 : *The production of recombinant human growth hormone (cDNA–Complementary DNA, hGH–Human growth hormone)*

3. Alteplase (Rx) - Activase -Tissue Plasminogen Activator

Tissue Plasminogen Activator (tPA) is a naturally occurring protease enzyme that helps to dissolve blood clots. tPA is a boon for patients suffering from thrombosis. The majority of natural deaths worldwide are due to a blockade of cerebral or coronary artery by a blood clot, technically called as thrombus. The phenomenon of thrombus blockage of blood vessels is referred to as thrombosis.

Chemically, thrombus consists of a network of fibrin, formed from the fibrinogen. In the normal circumstances, plasmin degrades fibrin and dissolves blood clots. This plasmin is actually produced by activation of plasminogen by tissue plasminogen activator (Fig. 15.3)

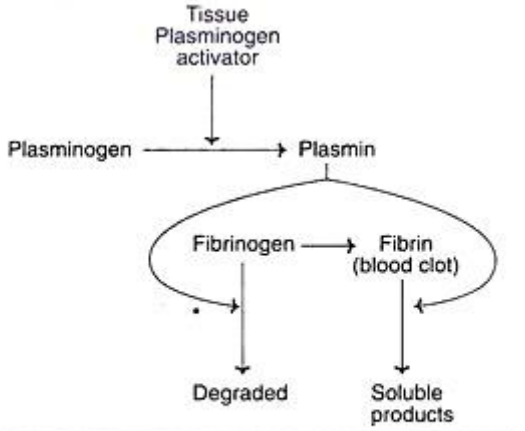


Fig. 15.3 : Role of tissue plasminogen activator in dissolving blood clots.

The natural biological systems is however, not that efficient to remove the blood clots through this machinery. Tissue plasminogen activator is very useful as a therapeutic agent in dissolving blood clots (thrombi) by activating plasminogen. By removing the arterial, thrombi, the possible damage caused by them on heart and brain could be reduced.

Production of recombinant tPA:

DNA technologists synthesized the complementary DNA (cDNA) molecule for tissue plasminogen activator. This cDNA was then attached to a synthetic plasmid and introduced into mammalian cells (Fig. 15.4). They were cultured and tPA-producing cells were selected by using methotrexate to the medium.

tPA-producing cells were transferred to an industrial tank (fermenter). tPA, secreted into the culture medium, is isolated for therapeutic purpose. It may be noted here that tPA was the first pharmaceutical product to be produced by mammalian cell culture.

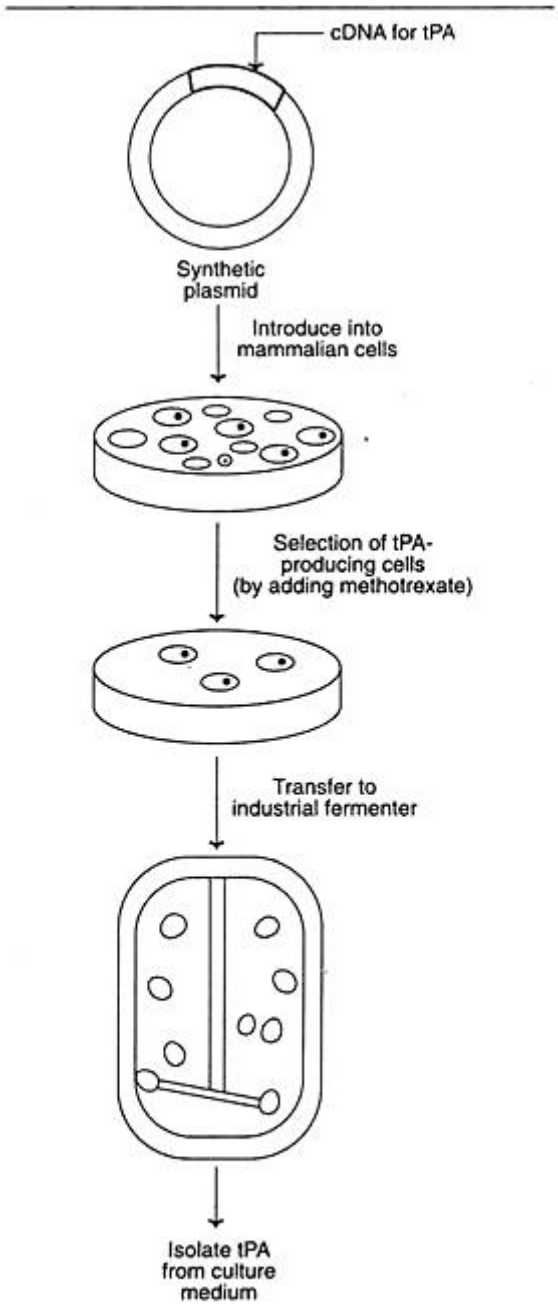


Fig. 15.4 : The production of recombinant tissue plasminogen activator (tPA).

Recombinant tPA has been in use since 1987 for treatment of patients with acute myocardial infarction or stroke. Gene-tech was the first to market tPA with a trade name ***Activase.***

Activase is used to dissolve blood clots that have formed in the blood vessels. It is used immediately after symptoms of a heart attack occur to improve patient survival. It is also used after symptoms of a stroke and to treat blood clots in the lungs (pulmonary embolism).

4. Hybridoma technology - Monoclonal antibodies (mAbs)

What is Hybridoma technology?

Hybridoma technology is a well-established method to produce monoclonal antibodies (mAbs) specific to antigens of interest. Hybridoma cell lines are formed via fusion between a short-lived antibody-producing B cell and an immortal myeloma cell.

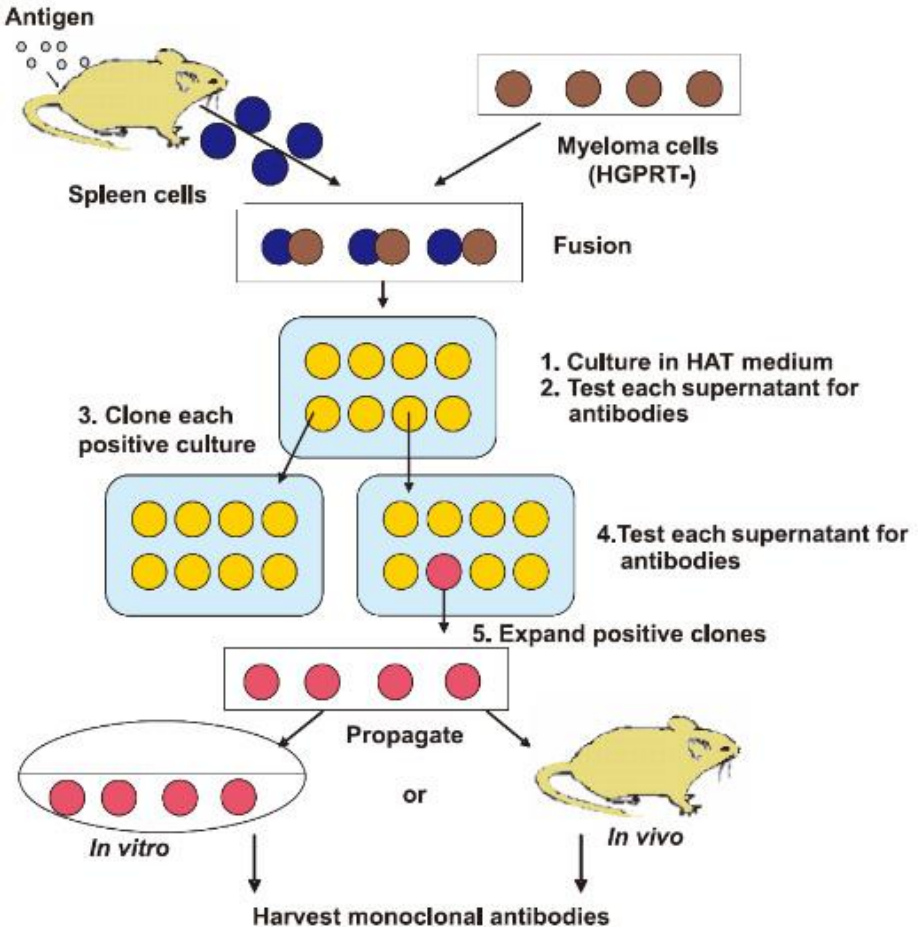
Hybridoma technology was discovered in 1975 by two scientists, Georges Kohler and Cesar Milstein. They wanted to create immortal hybrid cells by fusing normal B cells from immunized mice with their myeloma cells.

Their discovery is considered one of the greatest breakthroughs in the field of biotechnology. For the past decades, hybridomas have fuelled the discovery and production of antibodies for a multitude of applications.

Steps Involved in Hybridoma Technology

Hybridoma technology is composed of several technical procedures, including antigen preparation, animal immunization, cell fusion, hybridoma screening and sub cloning, as well as characterization and production of specific antibodies.

mAb generation by the hybridoma approach requires knowledge of multiple disciplines and practice of versatile technical skills, ranging from animal handling, immunology to cellular and molecular biology. Generation and identification of high-quality hybridoma clones is a comprehensive and labor-intensive process, and requires months of work during the time frame from immunization to specific hybridoma identification.



1) Cell fusion

Polyethylene glycol (PEG) and electro fusion are commonly used to induce cell fusion in hybridoma production. PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei. This hetero karyon retains these nuclei until the nuclear membranes dissolve before mitosis. Electrofusion joins the membranes of neighbouring cells by the application of a pulsed electrical field. Electrofusion is more efficient than PEG and the results are reproducible.

2) Hybridoma screening

Even in the most efficient hybridoma fusions, only about 1% of the starting cells are fused and only about 1 in 10^5 form viable hybrids. This leaves a large number of unfused cells still in culture. The cells from the immunized animal (antibody secreting cell) do not continue to grow in tissue culture and so do not confuse further work. However, the myeloma cells are well adapted to tissue culture and must be killed, which can be achieved by drug selection.

Commonly, the myeloma cells have a defective HGPRT enzyme (hypoxanthine-guanine phosphoribosyl transferase), blocking their ability to use the salvage pathway. These cells containing a non-functional HGPRT protein will die in HAT medium. Only the hybridoma cells have got the ability to divide and proliferate on the HAT medium because genome from the B-lymphocyte makes them HGPRT positive and genome from the myeloma cells they can divide indefinitely.

3) mAb production

Hybridoma antibodies can be produced in vitro and in vivo.

For production of monoclonal antibodies in vitro, hybridomas are expanded by transfer to 24 well tissue culture plates followed by 25 cm^2 flasks and a 75cm^2 flask containing suitable medium. The cell density is maintained between 10^5 and 10^6 cells/ml. Typical culture supernatants yield up to l00μg/ml of antibody, the exact amount depending upon the cell density and rate of growth. Culture in vitro provides a more pure preparation of antibody. Sino Biological can offer serum-free hybridoma production service by the use of serum-free medium.

For producing monoclonal antibodies in vivo, mice are primed by intra peritoneal injection with 10^5 - 10^7 hybridoma cells. The rate of growth of the resulting ascites tumour is in general very variable and can be from less than two or more than five weeks. The ascites fluid can be collected from an anaesthetized mouse. It is possible to obtain 10 ml of ascites fluid or more from a mouse by regular tapping. Ascites fluid will be contaminated with mouse imunoglobulins to a small extent and if a very pure antibody is required this may prove inconvenient.

Applications of Hybridoma Technology

mAb therapeutics

Compared with other biologics, mAbs are able to maintain an extremely high affinity towards their target. Due to this high affinity and specificity, researchers began investigating the therapeutic potential of mAbs as metabolic activators, inhibitors and immuno-modulators. While the first few US FDA-approved mAb therapeutics, such as muromonab-CD3, were generated solely in mice, it became evident that in order to avoid immune rejection, future

mAb-based therapeutics needed to undergo humanization. Since the approval of muromonab-CD3 in 1986, the FDA has approved approximately 80 more mAb therapeutics for diseases ranging from autoimmune disorders, to inflammatory diseases, HIV and cancer. Interestingly, despite the discovery of combinatorial display libraries in 1984 as an alternative mAb discovery platform, the majority of these mAb therapeutics were originally discovered using hybridoma technology in either fully murine or humanized mice. The reason for this preference is likely attributed to the natural ability of the murine immune system to generate highly specific mAbs that elicit strong constant domain functionality with limited immuno reactivity after humanization.

5. Interferons / Intron a:

Interferon is an antiviral substance, and is the first line of defense against viral attacks. The term interferon has originated from the interference of this molecule on virus replication. It was originally discovered in 1957 by Alick Isaacs and Jean Lindemann and was considered to be a single substance.

It is now known that interferon actually consists of a group of more than twenty substances with molecular weights between 20,000-30,000daltons. All the interferons are proteins in nature and many of them are glycoproteins. They are broadly categorized into three groups based on their structure and function,

- ✓ Interferon-α (IFN-α)
- ✓ Interferon-β (IFN-β)
- ✓ Interferon-y (IFN-y)

Interferon's (IFNs) are proteins made and released by host cells in response to the presence of pathogens such as viruses, bacteria, parasites or tumour cells. They allow for communication between cells to trigger the protective defences of the immune system that eradicate pathogens or tumours.

IFNs belong to the large class of glycoproteins known as cytokines. Interferons are named after their ability to "interfere" with viral replication within host cells.

IFNs have other functions: they activate immune cells, such as natural killer cells and macrophages; they increase recognition of infection or tumour cells by up-regulating antigen presentation to T lymphocytes; and they increase the ability of uninfected host cells to resist new infection by virus. Certain symptoms, such as aching muscles and fever, are related to the production of IFNs during infection.

About ten distinct IFNs have been identified in mammals; seven of these have been described for humans. They are typically divided among three IFN classes: Type I IFN, Type II IFN, and Type III IFN. IFNs belonging to all IFN classes are very important for fighting viral infections. Interferon type I: All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains. The type I interferons present in humans are IFN-α, IFN-β and IFN-ω.

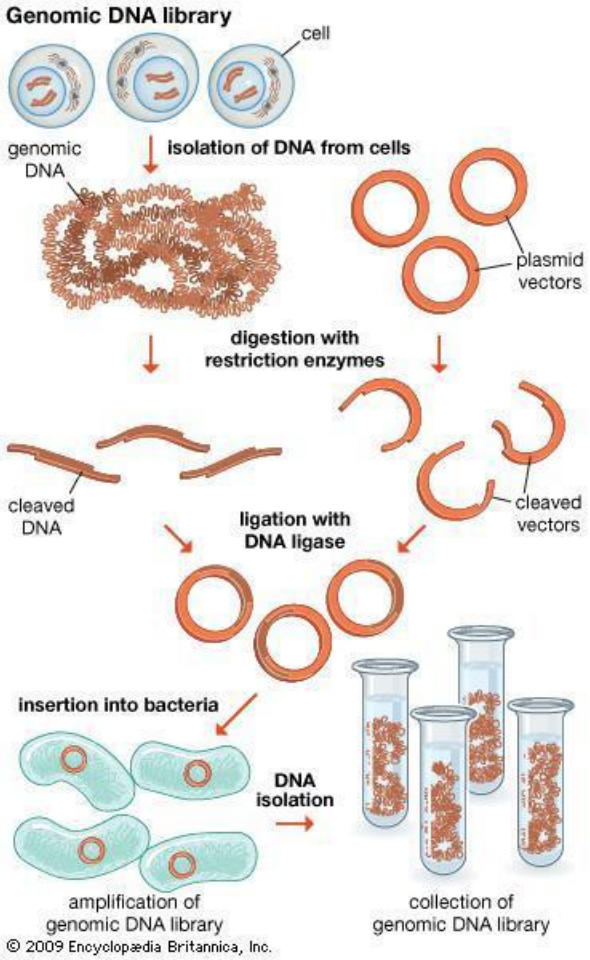
Isolation of interferons in the early years:

Blood was the only source of interferons earlier. The procedure was very tedious and the quantity of interferons isolated was very little. Thus, as much as 50,000 litres of human blood was required to get just 100 mg of interferons. Therefore, it was very difficult to conduct research or use interferons for therapeutic purposes.

Now it is possible to produce interferons by r DNA technology at much cheaper rate.

Production of recombinant interferons:

The complementary DNA (cDNA) was synthesized from the mRNA of a specific interferon. This is inserted to a vector (say plasmid) which is introduced into *E. coli* or other cells. The interferon can be isolated from the culture medium. This is the basic mechanism of producing recombinant interferons. The production of interferons is relatively less in bacterial hosts, although *E. coli* was the first to be used. This is mainly because most interferons are glycoproteins in nature and bacteria do not possess the machinery for glycosylation of proteins.



6. Recombivax HB (Hepatitis B)

A vaccine is a biological preparation that improves immunity to a particular disease. Injection of a killed microbe in order to stimulate the immune system against the microbe, thereby preventing disease.

Vaccine types:

- Live, attenuated vaccines
- Inactivated vaccine
- Subunit vaccine
- Toxoid vaccine
- Conjugated vaccine
- DNA vaccine
- **Recombinant vector vaccines**

Recombinant vector vaccines

Vaccine antigens may also be produced by genetic engineering technology. These products are sometimes referred to as recombinant vaccines

- **Subunit vaccines:**

These are the components of the pathogenic organisms. Subunit vaccines include protein, peptides and DNA.

- **Attenuated recombinant vaccines:**

These are the genetically modified pathogenic organisms that are made non-pathogenic and used as vaccines.

- **Vector recombinant vaccines:**

These are the genetically modified viral vectors that can be used as vaccines against certain pathogens.

Hepatitis B

Hepatitis B is a widespread disease in man. It primarily affects liver causing chronic hepatitis, cirrhosis and liver.

The gene encoding for hepatitis B surface antigen (HBs Ag) has been identified. The HBs Ag vaccine as a subunit vaccine is produced by cloning HBs Ag gene in yeast cells (*Sacchromyces cerevisiae*).

General features of nucleic acid of Hepatitis B Virus

HB virus has been identified as a 42-nm particle containing a double stranded circular DNA molecule of about 3Kb size. DNA genome has a relative molecular mass of approximately 2 X 10.

Plasma of human has been detected to have varying amount of HB antigens. Three types of viral coat proteins are recognized to be antigenic

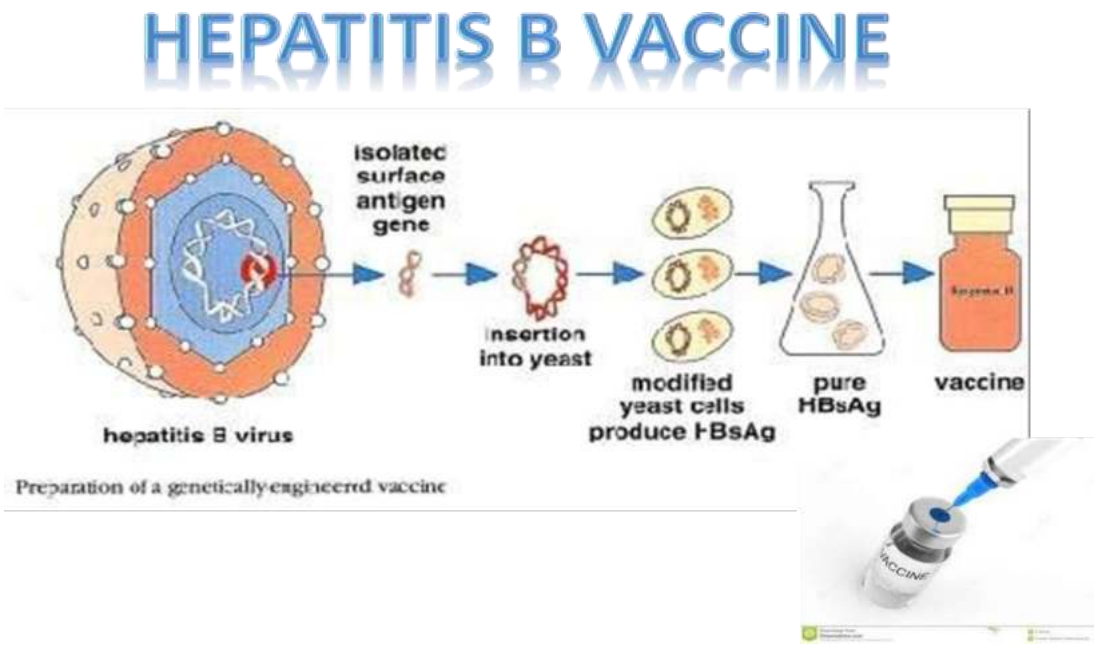
- viral surface antigen (HBs Ag)
- viral core antigen (HBc Ag)
- the e-antigen (HBe Ag)

Surface antigen HBs AG is found as 18-22 nm spherical or tubular form particles. Recently HBs Ag gene or it's subunits are used for the production of recombinant Hepatitis B vaccine.

General steps for Recombinant Hepatitis B Vaccine production

Production of these genes is needed in order to get production of vaccines on a large scale. A general procedure for the production of recombinant Hepatitis B vaccines are described here

1. HBs antigen producing gene is isolated from the HB virus by normal isolation process (cell lysis, protein denaturation, precipitation, centrifugation and drying).
2. A plasmid DNA is extracted from a bacterium- E.coli and is cut with restriction enzyme- Eco RI forming the plasmid vector
3. The isolated HBs antigen producing gene is located and inserted into the bacterial plasmid vector on forming the recombinant DNA.
4. This recombinant DNA, containing the target gene, is introduced into a yeast cell forming the recombinant yeast cell.
5. The recombinant yeast cell multiplies in the fermentation tank and produces the HBs antigens.
6. After 48 hours, yeast cells are ruptured to free HBs Ag. The mixture is processed for extraction.
7. The HBs antigens are purified.
8. HBs Ag are combined with preserving agent and other ingredients and bottled. Now it is ready for vaccination in humans.



Active & Passive Immunizations
Vaccine Preparation, Standardization & Storage
Vaccines-of BCG, Cholera, Smallpox, Polio, Typhus, Tetanus Toxoid, Immuno serum

Vaccine is an antigenic preparation which is used to attain immunity or resistance against infectious microorganisms. It contains an inactivated germ or a weakened (attenuated) form of live germ or a small part of the germ that acts as antigen to stimulate the immune response.

An ideal vaccine should be able to elicit the following:

1. Activate the antigen presenting cells to initiate antigen processing and produce interleukins.
2. Activate T-lymphocytes and B-lymphocytes to produce huge number of memory cells.
3. Produce T-helper cells and cytotoxic T-cells.
4. Persistence of antigen in dendritic follicular cells of lymphoid tissue where the B-memory cells produce antibody secreting cells which produce antibodies.

The immune system identifies the dead or harmless organisms (antigen) and responds by producing antibodies which bind to the disease causing organism and effectively destroy them. In addition, certain specialized cells called "memory cells" are produced that remain in circulating system throughout the life of the host. A subsequent infection with the same disease-causing agent evokes immediate protective immune response, inactivates the disease causing agents and thus prevents the symptoms of the disease. The person is now said to be immune from infection.

Types of vaccines: Vaccines are mainly of any of the following types.

1. Killed whole organism
2. Attenuated organism
3. Toxoids
4. Surface molecules
5. Recombinant vector vaccines
6. DNA vaccines

1. **Killed whole organism:** The killed (inactivated) vaccines can stimulate a protective immune response without causing infection. The entire organism is killed or inactivated with chemicals such as formaldehyde to make it harmless.

   Eg: Typhoid vaccine, Inactivated Polio Vaccine (IPV), Inactivated Influenza Vaccine, etc.

2. **Attenuated organism:** In this type of vaccine, the organisms are weakened or attenuated by growing them over and over again (also referred as passing) under nourishing conditions to reduce their pathogenicity or disease causing ability.

   Eg: MMR (measles, mumps and rubella) vaccine, oral polio vaccine (OPV), varicella (chicken pox) vaccine, BCG (Bacillus Calmette Guerin) vaccine.

3. **Toxoids:** These are proteinaceous toxins liberated by the bacteria which are rendered harmless by treating with heat or with formaldehyde to denature the protein. It still retains the capacity to stimulate the formation of toxin antibodies or antitoxins.

   Eg: Diphtheria Toxoid Vaccine, Tetanus Toxoid Vaccine.

4. **Surface molecules:** When antibodies bind to the surface of invading pathogen, the destruction of pathogen is triggered, thus protecting the host. Some vaccines contain

purified surface molecules that stimulate the production of antibodies. These are also referred to as component vaccines.

Eg:

a. The *influenza* vaccine contains purified surface molecules, i.e. haemagglutinins or inactivated virus particles from three major strains A, B and C.

b. The hepatitis B vaccine is a recombinant antigen vaccine produced by using recombinant DNA technology. The gene encoding the protein of hepatitis B virus surface antigen or HBsAg can e expressed in yeast cells or *E.coli* and growing them in large fermenters. The cells are cultured which then undergo disruption to release the recombinant HBsAg, which is purified by conventional biochemical procedures.

c. The human papilloma virus vaccine too is a recombinant antigen vaccine produced by using recombinant DNA technology. The gene encoding the capsid proteins of human papilloma virus are expressed in yeast cells. The recombinant capsid proteins are incorporated in a vaccine.

d. The vaccines for *Strepococcus pneumniae* and *Neisseria meningitides* consists of purified capsular polysaccharides.

5. **Recombinant vector vaccines:** Attenuated viruses or bacteria serve as a carrier to insert genes coding for antigens of virulent pathogens. The vaccinia virus with genome of 200 genes is used as a vector. The procedure is as follows:

   The plasmid is first cleaved adjacent to the vaccinia promoter using a restriction enzyme where the DNA encoding the antigen from pathogen is inserted. The plasmid is then ligated. The vaccinia virus and recombinant plasmids are allowed to grow in tissue culture cells. Incubation of cells results in insertion of antigen gene and promoter into the vaccinia virus by hologous recombination. Now, the genetically engineered virus can produce high levels of inserted DNA for the antigens.

6. **DNA vaccines:** The plasmid DNA encoding antigen from pathogen is inserted directly into the muscle cells. The inserted DNA induces the production of antigen molecules, which when displayed on the cell surface stimulate both humoral and cell mediated immunity.

   Merits:

   - ✓ A single injection of DNA vaccine is enough to provide immunity for a long duration.
   - ✓ The genes encoding the antigens form several pathogens can be combined and given as a single injection, thus protecting the animal from multiple diseases.
   - ✓ Special care need not be taken while handling DNA vaccine as these are considered to be extremely stable.

1. Poliomyelitis Vaccine

Poliomyelitis is a dreaded disease caused by poliovirus that may attack DNA and cause paralysis. Three strains of poliomyelitis virus with distinct antigenic properties are type I, II and III. The general methodology for the preparation of polio vaccine is as below. The three strains are cultured separately in suspended or fixed cell cultures of Rhesus monkey kidney tissue that have been examined thoroughly for any disease both before and after the death of the animal. Poliomyelitis vaccine is of two types.

1. **Inactivated (killed) vaccine (Salk type polio vaccine):**
   This type of vaccine was developed by an American virologist, Dr. Jonas Salk. The viral suspension obtained in the tissue culture is tested for the presence of correct strain of poliomyelitis virus. To make it free from contaminating agents like viruses, bacteria and fungi, this suspension is passed through a series of filters with decreasing thickness.
   This is followed by inactivation of the viral particles using 0.01%formaldehyde solution at pH, temperature and with magnetic stirring for 10-13 days to ensure that no active virus remains.
   The univalent vaccines are mixed to form the trivalent product. This again is tested for the presence of contaminants, if any. This is followed by the addition of sodium metabisulphite to neutralize the formaldehyde and thiomersal to form products that are toxic to the virus.
2. **Attenuated (avirulent) vaccine (Sabin type oral vaccine)**
   This type of vaccine was developed by Dr. Albert Sabin, an American scientist. The methodology for developing is similar to the Salk type vaccine with the exception that-
   1. The strains are developed by repeated passages in monkey kidney cells.
   2. Inactivation of viral particles is not involved.
   3. Through testing for contaminants as well as the presence of virulent forms of poliomyelitis virus is essential.

Storage: The vaccine must be stored at -20^0C or below under frozen state for long term storage upto 2 years. When the vaccine is thawed (liquid form), it must be kept in refrigerator at 2^0C to 8^0C. Vaccine if stored at 2^0C to 8^0C shall remain good for only 4-6 months.

2. Tetanus

Tetanus vaccine, also known as **tetanus toxoid** (**TT**), is an inactive vaccine or exotoxins released by *Clostridium tetani* used to prevent tetanus (lock jaw) caused by Clostridium tetani.

Clostridium tetani is a spore-forming anaerobic bacillus. Spores are present in the environment, particularly in the soil of warm and moist areas, and may be carried in the intestinal tracts of humans and animals.

Under favourable anaerobic conditions, such as in dirty, necrotic wounds, this ubiquitous bacillus may produce an extremely potent neurotoxin which causes muscular stiffness and spasm. Characteristically, early spasms of the facial muscles ("lockjaw") are followed by spasm of the back muscles and sudden, generalized tonic seizures and causes disease.

Preparation of vaccine:

Immunity to tetanus is antibody-mediated and depends upon the ability of antitoxin antibodies to neutralize tetanus toxin. Recovery from clinical tetanus does not result in protection against future disease, and immunity can be obtained only by active or passive immunization such as vaccination, immunoglobulin therapy, or transfer of maternal antibodies through the placenta.

Tetanus vaccine is prepared by the exotoxins released from anaerobic bacteria, *Clostridium tetani* cultured in a suitable growth media. The powerful nerve toxin produced by this bacterium causes tetanus.
The exotoxins converted to a taxoid by treating with formaldehyde solution. This is followed by the addition of antimicrobial preservative to the final product.

Toxigenic strains of *C. tetani* are grown in liquid media; the toxin is purified, and then inactivated by treatment with formaldehyde to produce the toxoid antigen.

After purification and sterilization, tetanus toxoid is formulated with aluminum or calcium salts and administered by intramuscular injection.

Tetanus toxoid vaccine is available as a single antigen vaccine (TT), in combination with diphtheria toxoid in infant and adult doses (DT and Td), and in combination with diphtheria and whole-cell or acellular pertussis (DTP). DTP-containing multi-antigen vaccines (with Hep B, Hib, or IPV) are increasingly in use in national immunization campaigns.

Standardization and storage:

The prepared vaccines were checked for the content of exotoxins per ml and it should meet the standards given by WHO and are stored by freeze drying.

During childhood five doses are recommended, with a sixth given during adolescence. Additional doses every 10 years are recommended. After three doses almost everyone is initially immune.

In those who are not up to date on their tetanus immunization a booster should be given within 48 hours of an injury. In those with high risk injuries who are not fully immunized tetanus antitoxin may also be recommended.

3. Cholera

Cholera is a serious infection of the intestine caused by the bacterium, *Vibrio cholerae.* Its symptoms include severe vomiting and diarrhoea leading to dehydration. Vaccination against this disease is effective for 8-9 months.

Method of preparation: The vaccine for cholera is a type of killed bacterial vaccine.

Cholera vaccine is prepared form two classical types of *Vibrio cholera,* Inaba and Ogawa.

The bacterial cells are cleared of contaminants (if any), inoculated in a culture medium (solid or liquid) and incubated for about 3 days in appropriate conditions.

When the cells are grown in a solid culture medium, the culture is washed with saline to remove the cells and then centrifuged to remove agar that would have got detached during the washing process.

When the cells are grown in liquid culture media, following centrifugation, the cells settle at the bottom of the centrifuge tube. The cells are washed and centrifuged twice or thrice to remove the broth constituents.

Then the organisms are killed by heat treatment or by the addition of bactericide such as phenol or formaldehyde.

The bacterial cells per ml are then estimated by either Helber cells, brown tubes or photoelectric nephlometer.

Storage: A suitable preservative is added to the final preparation and stored at a temperature between 2°C and 8°C.

4. BCG Vaccine

BCG vaccine is a type of attenuated bacterial vaccine and is used as a preventive vaccine against tuberculosis in humans.

Preparation of BCG vaccine:

This vaccine consists of attenuated strains of *Mycobacterium bovis.* The basic principle underlying the use of BCG vaccine is to develop resistance in the subject by producing a tuberculosis infection. However, being attenuated, the mycobacterium is only capable of giving rise to local lesions and lymphadenopathy which tends to regress with time.

BCG Vaccine for percutaneous use is an attenuated, live culture preparation of the Bacillus of Calmette and Guerin (BCG) strain of *Mycobacterium bovis.*

The medium in which BCG organism is grown for preparation of the freeze-dried cake is composed of the following ingredients: glycerin, asparagine, citric acid, potassium phosphate, magnesium sulfate, and iron ammonium citrate.

The final preparation prior to freeze drying also contains lactose.

Standardization and storage:

The freeze-dried BCG preparation is delivered in vials, each containing 1 to 8 x 108 colony forming units (CFU) of BCG which is equivalent to approximately 50 mg wet weight. Determination of *in-vitro* potency is achieved through colony counts derived from a serial dilution assay.

Intradermal guinea pig testing is also used as an indirect measure of potency. Reconstitution requires addition of Sterile Water for Injection, U.S.P. at 4–25°C (39-77°F). For an adult dosage, 1mL of Sterile Water for Injection, U.S.P., should be added to one vial of vaccine. For a pediatric dosage, 2mL of Sterile Water for Injection, U.S.P., should be added to one vial of vaccine

No preservatives have been added.

Generally infants less than 6 months of age are administered the BCG vaccine. Usually, prior to administration, the individual is subjected to tuberculin testing. However, such testing is not necessary in infants.

0.1 ml of BCG vaccine is administered intradermally into the deltoid region only when two consecutive pre testings with purified protein derivative (PPD) gives negative results. At the site of injection, a papule develops, which within 1-1½ month transform into an ulcer. This ulcer may eventually undergo healing but the papule is evident for about a year. After vaccination, the subject tests positive for tuberculin skin test which indicates that the BCG vaccine has conferred immunity.

5. Typhus vaccine

Typhus (spotted fever) is a type of infection caused by rickettsiae, a non motile spherical or rod shaped bacteria that cannot reproduce outside of their hosts. It is characterized by headache, skin rash, prolonged high fever and delirium.

Preparation of vaccine: The typhus vaccine is prepared in the following manner.

- ✓ The fertile eggs are first incubated for one week.
- ✓ The virulent forms of rickettsiae are injected into the yolk sac of fertile eggs.
- ✓ After about 13 days of injection, the yolk sacs from the eggs are removed under sterile conditions and homogneized to free the rickettsiae.
- ✓ The homogenized suspension is treated with 0.2 to 0.5% w/w of yolk sac tissue.
- ✓ Further purification is done by treating with ether or trichlorotrifluoroethane.
- ✓ The aqueous layer of the suspension is collected and treated with a bactericide, thiomersal.
- ✓ The resulting preparation is collected in sterile containers under aseptic conditions and sealed properly.

Storage conditions:

Typhoid vaccine must be stored in the refrigerator at a temperature between 2^0 and 8^0C (35.6 and 46.4 degrees F) at all times. If the vaccine is left at room temperature, it will lose its effectiveness. Therefore, remember to replace unused vaccine in the refrigerator between doses.

6. Small pox vaccine

Small pox is caused by *Variola virus*. There are two forms of the virus, *Variola major* and *Variola minor.*

Preparation of vaccine:

Unlike other vaccines, small pox vaccine is made from virus which is not responsible for causing small pox.

The vaccine is made from a virus called *Vaccinia*, which is a pox virus, similar to smallpox, but less harmful. It protects people from smallpox by helping their bodies develop immunity to smallpox.

The smallpox vaccine contains live *Vaccinia* virus, not a killed or weakened virus like many other vaccines.

Traditional methods of preparing small pox vaccine entail passage of Vaccinia virus through living mammalian hosts which have difficulty in obtaining free of bacterial contaminants.

The newer methods include the development of vaccine in chick embryos. A 12 day old chick embryo should be decapitated, eviscerated and cut into pieces.

Then these pieces are washed in phosphate buffered saline and digested with Trypsin. Then the cells are washed free of trypsin by centrifugation process.

The embryonic cells seeded with vaccinia virus are harvestsed and cultured in suitable medium which mainly include earle's saline, hartley's digest broth, native horse serum, L-cysteine hydrochloride, lactalbumen hydrolysate, penicillin, streptomycin etc.

The cells are to be broken by homogenization at 4^0C to release virus for a shorter duration at high rpm.

Then the amount of virus per ml has to be estimated.

For most people with healthy immune systems, live virus vaccines are effective and safe. Sometimes a person getting a live virus vaccine experiences mild symptoms such as rash, fever, and head and body aches. In certain groups of people, complications from the vaccinia virus can be severe.

Other live virus vaccines currently used include measles, mumps, rubella, and chickenpox.

Standardization and storage:

Smallpox vaccine should always be protected from heat, and should never be frozen.

Smallpox vaccine must be kept at appropriate temperatures to retain effectiveness. There are two major problems in storing and handling vaccines which can rapidly reduce their potency: either 1) freezing vaccines that should not be frozen, or 2) letting infectious (live) vaccines warm.

Smallpox vaccine should never be frozen and should be kept between 2°– 8°C (36° – 46°F).

After its preparation, it was standardized according to the guidelines given by WHO. The effective amount of virus per ml is calculated and stored in ampoules under freeze drying. Smallpox vaccination can protect the host from smallpox for about 3 to 5 years. For long-term protection, a booster vaccination is needed.

The vaccine has been effective in preventing smallpox infection in 95% of those vaccinated. In addition, the vaccine was proven to prevent or substantially lessen infection when given within a few days after a person was exposed to the *Variola* virus.

7. Immunizing sera

Immune globulins from human serum have been in use for many years to protect against the emergence of diseases. Immunologists have devised a method for the separation of γ-globulin fractions from serum by precipitation with cold ethanol. This method was called "Cohn fractionation".

The plasma or serum is collected from healthy donors, pooled and used to prepare immune serum globulin (ISG) or human normal immunoglobulin (HNI). If the donors have recovered from a specific infection, or have been immunized against the disease with large doses of Toxoids, then it is designated accordingly.

Eg: Tetanus immune globulin, Hepatitis B immune globulin, Varicella Zoster immune, Rabies immune globulin, etc.

For instance, tetanus immune globulin a sterile solution of globulins obtained from the plasma of adults hyperimmunized with tetanus toxoid. It is effective against patients who suffer from wounds contaminated with *Clostidium tetani.*

Following the serum collection from donors, the blood cells are removed and returned to the donor. This process is called plasmapheresis. The portion of the serum containing antibody globulins is precipitated by cold ethanol.

The preparation should have the following characteristics.

- ✓ It is free from infectious agents.
- ✓ It is highly concentrated.
- ✓ The preparation is stable for many years.
- ✓ Peak blood levels of IgG can be obtained within 2 days of intramuscular injection.

The intravenous immune globulin preparation includes alcohol precipitation, fractionation using polyethylene glycol (PEG) or ion exchangers, acidification of the preparation to pH 4.0 - 4.5, treatment with pepsin or trypsin and finally stabilization of the preparation using maltose or glycine to prevent aggregation of globulins.

The preparation should be stored under normal refrigeration between 2°C to 8°C.

Active & Passive Immunizations
Vaccine Preparation, Standardization & Storage
Vaccines-of BCG, Cholera, Smallpox, Polio, Typhus, Tetanus Toxoid, Immuno serum

Vaccine is an antigenic preparation which is used to attain immunity or resistance against infectious microorganisms. It contains an inactivated germ or a weakened (attenuated) form of live germ or a small part of the germ that acts as antigen to stimulate the immune response.

An ideal vaccine should be able to elicit the following:

1. Activate the antigen presenting cells to initiate antigen processing and produce interleukins.
2. Activate T-lymphocytes and B-lymphocytes to produce huge number of memory cells.
3. Produce T-helper cells and cytotoxic T-cells.
4. Persistence of antigen in dendritic follicular cells of lymphoid tissue where the B-memory cells produce antibody secreting cells which produce antibodies.

The immune system identifies the dead or harmless organisms (antigen) and responds by producing antibodies which bind to the disease causing organism and effectively destroy them. In addition, certain specialized cells called "memory cells" are produced that remain in circulating system throughout the life of the host. A subsequent infection with the same disease-causing agent evokes immediate protective immune response, inactivates the disease causing agents and thus prevents the symptoms of the disease. The person is now said to be immune from infection.

Types of vaccines: Vaccines are mainly of any of the following types.

1. Killed whole organism
2. Attenuated organism
3. Toxoids
4. Surface molecules
5. Recombinant vector vaccines
6. DNA vaccines

1. **Killed whole organism:** The killed (inactivated) vaccines can stimulate a protective immune response without causing infection. The entire organism is killed or inactivated with chemicals such as formaldehyde to make it harmless.

   Eg: Typhoid vaccine, Inactivated Polio Vaccine (IPV), Inactivated Influenza Vaccine, etc.

2. **Attenuated organism:** In this type of vaccine, the organisms are weakened or attenuated by growing them over and over again (also referred as passing) under nourishing conditions to reduce their pathogenicity or disease causing ability.

   Eg: MMR (measles, mumps and rubella) vaccine, oral polio vaccine (OPV), varicella (chicken pox) vaccine, BCG (Bacillus Calmette Guerin) vaccine.

3. **Toxoids:** These are proteinaceous toxins liberated by the bacteria which are rendered harmless by treating with heat or with formaldehyde to denature the protein. It still retains the capacity to stimulate the formation of toxin antibodies or antitoxins.

   Eg: Diphtheria Toxoid Vaccine, Tetanus Toxoid Vaccine.

4. **Surface molecules:** When antibodies bind to the surface of invading pathogen, the destruction of pathogen is triggered, thus protecting the host. Some vaccines contain

purified surface molecules that stimulate the production of antibodies. These are also referred to as component vaccines.

Eg:

a. The *influenza* vaccine contains purified surface molecules, i.e. haemagglutinins or inactivated virus particles from three major strains A, B and C.

b. The hepatitis B vaccine is a recombinant antigen vaccine produced by using recombinant DNA technology. The gene encoding the protein of hepatitis B virus surface antigen or HBsAg can e expressed in yeast cells or *E.coli* and growing them in large fermenters. The cells are cultured which then undergo disruption to release the recombinant HBsAg, which is purified by conventional biochemical procedures.

c. The human papilloma virus vaccine too is a recombinant antigen vaccine produced by using recombinant DNA technology. The gene encoding the capsid proteins of human papilloma virus are expressed in yeast cells. The recombinant capsid proteins are incorporated in a vaccine.

d. The vaccines for *Strepococcus pneumniae* and *Neisseria meningitides* consists of purified capsular polysaccharides.

5. **Recombinant vector vaccines:** Attenuated viruses or bacteria serve as a carrier to insert genes coding for antigens of virulent pathogens. The vaccinia virus with genome of 200 genes is used as a vector. The procedure is as follows:

   The plasmid is first cleaved adjacent to the vaccinia promoter using a restriction enzyme where the DNA encoding the antigen from pathogen is inserted. The plasmid is then ligated. The vaccinia virus and recombinant plasmids are allowed to grow in tissue culture cells. Incubation of cells results in insertion of antigen gene and promoter into the vaccinia virus by hologous recombination. Now, the genetically engineered virus can produce high levels of inserted DNA for the antigens.

6. **DNA vaccines:** The plasmid DNA encoding antigen from pathogen is inserted directly into the muscle cells. The inserted DNA induces the production of antigen molecules, which when displayed on the cell surface stimulate both humoral and cell mediated immunity.

   Merits:

   - ✓ A single injection of DNA vaccine is enough to provide immunity for a long duration.
   - ✓ The genes encoding the antigens form several pathogens can be combined and given as a single injection, thus protecting the animal from multiple diseases.
   - ✓ Special care need not be taken while handling DNA vaccine as these are considered to be extremely stable.

1. Poliomyelitis Vaccine

Poliomyelitis is a dreaded disease caused by poliovirus that may attack DNA and cause paralysis. Three strains of poliomyelitis virus with distinct antigenic properties are type I, II and III. The general methodology for the preparation of polio vaccine is as below. The three strains are cultured separately in suspended or fixed cell cultures of Rhesus monkey kidney tissue that have been examined thoroughly for any disease both before and after the death of the animal. Poliomyelitis vaccine is of two types.

1. **Inactivated (killed) vaccine (Salk type polio vaccine):**
   This type of vaccine was developed by an American virologist, Dr. Jonas Salk. The viral suspension obtained in the tissue culture is tested for the presence of correct strain of poliomyelitis virus. To make it free from contaminating agents like viruses, bacteria and fungi, this suspension is passed through a series of filters with decreasing thickness.
   This is followed by inactivation of the viral particles using 0.01%formaldehyde solution at pH, temperature and with magnetic stirring for 10-13 days to ensure that no active virus remains.
   The univalent vaccines are mixed to form the trivalent product. This again is tested for the presence of contaminants, if any. This is followed by the addition of sodium metabisulphite to neutralize the formaldehyde and thiomersal to form products that are toxic to the virus.
2. **Attenuated (avirulent) vaccine (Sabin type oral vaccine)**
   This type of vaccine was developed by Dr. Albert Sabin, an American scientist. The methodology for developing is similar to the Salk type vaccine with the exception that-
   1. The strains are developed by repeated passages in monkey kidney cells.
   2. Inactivation of viral particles is not involved.
   3. Through testing for contaminants as well as the presence of virulent forms of poliomyelitis virus is essential.

Storage: The vaccine must be stored at -20^0C or below under frozen state for long term storage upto 2 years. When the vaccine is thawed (liquid form), it must be kept in refrigerator at 2^0C to 8^0C. Vaccine if stored at 2^0C to 8^0C shall remain good for only 4-6 months.

2. Tetanus

Tetanus vaccine, also known as **tetanus toxoid** (**TT**), is an inactive vaccine or exotoxins released by *Clostridium tetani* used to prevent tetanus (lock jaw) caused by Clostridium tetani.

Clostridium tetani is a spore-forming anaerobic bacillus. Spores are present in the environment, particularly in the soil of warm and moist areas, and may be carried in the intestinal tracts of humans and animals.

Under favourable anaerobic conditions, such as in dirty, necrotic wounds, this ubiquitous bacillus may produce an extremely potent neurotoxin which causes muscular stiffness and spasm. Characteristically, early spasms of the facial muscles ("lockjaw") are followed by spasm of the back muscles and sudden, generalized tonic seizures and causes disease.

Preparation of vaccine:

Immunity to tetanus is antibody-mediated and depends upon the ability of antitoxin antibodies to neutralize tetanus toxin. Recovery from clinical tetanus does not result in protection against future disease, and immunity can be obtained only by active or passive immunization such as vaccination, immunoglobulin therapy, or transfer of maternal antibodies through the placenta.

Tetanus vaccine is prepared by the exotoxins released from anaerobic bacteria, *Clostridium tetani* cultured in a suitable growth media. The powerful nerve toxin produced by this bacterium causes tetanus.
The exotoxins converted to a taxoid by treating with formaldehyde solution. This is followed by the addition of antimicrobial preservative to the final product.

Toxigenic strains of *C. tetani* are grown in liquid media; the toxin is purified, and then inactivated by treatment with formaldehyde to produce the toxoid antigen.

After purification and sterilization, tetanus toxoid is formulated with aluminum or calcium salts and administered by intramuscular injection.

Tetanus toxoid vaccine is available as a single antigen vaccine (TT), in combination with diphtheria toxoid in infant and adult doses (DT and Td), and in combination with diphtheria and whole-cell or acellular pertussis (DTP). DTP-containing multi-antigen vaccines (with Hep B, Hib, or IPV) are increasingly in use in national immunization campaigns.

Standardization and storage:

The prepared vaccines were checked for the content of exotoxins per ml and it should meet the standards given by WHO and are stored by freeze drying.

During childhood five doses are recommended, with a sixth given during adolescence. Additional doses every 10 years are recommended. After three doses almost everyone is initially immune.

In those who are not up to date on their tetanus immunization a booster should be given within 48 hours of an injury. In those with high risk injuries who are not fully immunized tetanus antitoxin may also be recommended.

3. Cholera

Cholera is a serious infection of the intestine caused by the bacterium, *Vibrio cholerae.* Its symptoms include severe vomiting and diarrhoea leading to dehydration. Vaccination against this disease is effective for 8-9 months.

Method of preparation: The vaccine for cholera is a type of killed bacterial vaccine.

Cholera vaccine is prepared form two classical types of *Vibrio cholera,* Inaba and Ogawa.

The bacterial cells are cleared of contaminants (if any), inoculated in a culture medium (solid or liquid) and incubated for about 3 days in appropriate conditions.

When the cells are grown in a solid culture medium, the culture is washed with saline to remove the cells and then centrifuged to remove agar that would have got detached during the washing process.

When the cells are grown in liquid culture media, following centrifugation, the cells settle at the bottom of the centrifuge tube. The cells are washed and centrifuged twice or thrice to remove the broth constituents.

Then the organisms are killed by heat treatment or by the addition of bactericide such as phenol or formaldehyde.

The bacterial cells per ml are then estimated by either Helber cells, brown tubes or photoelectric nephlometer.

Storage: A suitable preservative is added to the final preparation and stored at a temperature between 2°C and 8°C.

4. BCG Vaccine

BCG vaccine is a type of attenuated bacterial vaccine and is used as a preventive vaccine against tuberculosis in humans.

Preparation of BCG vaccine:

This vaccine consists of attenuated strains of *Mycobacterium bovis.* The basic principle underlying the use of BCG vaccine is to develop resistance in the subject by producing a tuberculosis infection. However, being attenuated, the mycobacterium is only capable of giving rise to local lesions and lymphadenopathy which tends to regress with time.

BCG Vaccine for percutaneous use is an attenuated, live culture preparation of the Bacillus of Calmette and Guerin (BCG) strain of *Mycobacterium bovis.*

The medium in which BCG organism is grown for preparation of the freeze-dried cake is composed of the following ingredients: glycerin, asparagine, citric acid, potassium phosphate, magnesium sulfate, and iron ammonium citrate.

The final preparation prior to freeze drying also contains lactose.

Standardization and storage:

The freeze-dried BCG preparation is delivered in vials, each containing 1 to 8 x 108 colony forming units (CFU) of BCG which is equivalent to approximately 50 mg wet weight. Determination of *in-vitro* potency is achieved through colony counts derived from a serial dilution assay.

Intradermal guinea pig testing is also used as an indirect measure of potency. Reconstitution requires addition of Sterile Water for Injection, U.S.P. at 4–25°C (39-77°F). For an adult dosage, 1mL of Sterile Water for Injection, U.S.P., should be added to one vial of vaccine. For a pediatric dosage, 2mL of Sterile Water for Injection, U.S.P., should be added to one vial of vaccine

No preservatives have been added.

Generally infants less than 6 months of age are administered the BCG vaccine. Usually, prior to administration, the individual is subjected to tuberculin testing. However, such testing is not necessary in infants.

0.1 ml of BCG vaccine is administered intradermally into the deltoid region only when two consecutive pre testings with purified protein derivative (PPD) gives negative results. At the site of injection, a papule develops, which within 1-1½ month transform into an ulcer. This ulcer may eventually undergo healing but the papule is evident for about a year. After vaccination, the subject tests positive for tuberculin skin test which indicates that the BCG vaccine has conferred immunity.

5. Typhus vaccine

Typhus (spotted fever) is a type of infection caused by rickettsiae, a non motile spherical or rod shaped bacteria that cannot reproduce outside of their hosts. It is characterized by headache, skin rash, prolonged high fever and delirium.

Preparation of vaccine: The typhus vaccine is prepared in the following manner.

- ✓ The fertile eggs are first incubated for one week.
- ✓ The virulent forms of rickettsiae are injected into the yolk sac of fertile eggs.
- ✓ After about 13 days of injection, the yolk sacs from the eggs are removed under sterile conditions and homogneized to free the rickettsiae.
- ✓ The homogenized suspension is treated with 0.2 to 0.5% w/w of yolk sac tissue.
- ✓ Further purification is done by treating with ether or trichlorotrifluoroethane.
- ✓ The aqueous layer of the suspension is collected and treated with a bactericide, thiomersal.
- ✓ The resulting preparation is collected in sterile containers under aseptic conditions and sealed properly.

Storage conditions:

Typhoid vaccine must be stored in the refrigerator at a temperature between 2^0 and 8^0C (35.6 and 46.4 degrees F) at all times. If the vaccine is left at room temperature, it will lose its effectiveness. Therefore, remember to replace unused vaccine in the refrigerator between doses.

6. Small pox vaccine

Small pox is caused by *Variola virus*. There are two forms of the virus, *Variola major* and *Variola minor.*

Preparation of vaccine:

Unlike other vaccines, small pox vaccine is made from virus which is not responsible for causing small pox.

The vaccine is made from a virus called *Vaccinia*, which is a pox virus, similar to smallpox, but less harmful. It protects people from smallpox by helping their bodies develop immunity to smallpox.

The smallpox vaccine contains live *Vaccinia* virus, not a killed or weakened virus like many other vaccines.

Traditional methods of preparing small pox vaccine entail passage of Vaccinia virus through living mammalian hosts which have difficulty in obtaining free of bacterial contaminants.

The newer methods include the development of vaccine in chick embryos. A 12 day old chick embryo should be decapitated, eviscerated and cut into pieces.

Then these pieces are washed in phosphate buffered saline and digested with Trypsin. Then the cells are washed free of trypsin by centrifugation process.

The embryonic cells seeded with vaccinia virus are harvestsed and cultured in suitable medium which mainly include earle's saline, hartley's digest broth, native horse serum, L-cysteine hydrochloride, lactalbumen hydrolysate, penicillin, streptomycin etc.

The cells are to be broken by homogenization at 4^0C to release virus for a shorter duration at high rpm.

Then the amount of virus per ml has to be estimated.

For most people with healthy immune systems, live virus vaccines are effective and safe. Sometimes a person getting a live virus vaccine experiences mild symptoms such as rash, fever, and head and body aches. In certain groups of people, complications from the vaccinia virus can be severe.

Other live virus vaccines currently used include measles, mumps, rubella, and chickenpox.

Standardization and storage:

Smallpox vaccine should always be protected from heat, and should never be frozen.

Smallpox vaccine must be kept at appropriate temperatures to retain effectiveness. There are two major problems in storing and handling vaccines which can rapidly reduce their potency: either 1) freezing vaccines that should not be frozen, or 2) letting infectious (live) vaccines warm.

Smallpox vaccine should never be frozen and should be kept between 2°– 8°C (36° – 46°F).

After its preparation, it was standardized according to the guidelines given by WHO. The effective amount of virus per ml is calculated and stored in ampoules under freeze drying. Smallpox vaccination can protect the host from smallpox for about 3 to 5 years. For long-term protection, a booster vaccination is needed.

The vaccine has been effective in preventing smallpox infection in 95% of those vaccinated. In addition, the vaccine was proven to prevent or substantially lessen infection when given within a few days after a person was exposed to the *Variola* virus.

7. Immunizing sera

Immune globulins from human serum have been in use for many years to protect against the emergence of diseases. Immunologists have devised a method for the separation of γ-globulin fractions from serum by precipitation with cold ethanol. This method was called "Cohn fractionation".

The plasma or serum is collected from healthy donors, pooled and used to prepare immune serum globulin (ISG) or human normal immunoglobulin (HNI). If the donors have recovered from a specific infection, or have been immunized against the disease with large doses of Toxoids, then it is designated accordingly.

Eg: Tetanus immune globulin, Hepatitis B immune globulin, Varicella Zoster immune, Rabies immune globulin, etc.

For instance, tetanus immune globulin a sterile solution of globulins obtained from the plasma of adults hyperimmunized with tetanus toxoid. It is effective against patients who suffer from wounds contaminated with *Clostidium tetani.*

Following the serum collection from donors, the blood cells are removed and returned to the donor. This process is called plasmapheresis. The portion of the serum containing antibody globulins is precipitated by cold ethanol.

The preparation should have the following characteristics.

- ✓ It is free from infectious agents.
- ✓ It is highly concentrated.
- ✓ The preparation is stable for many years.
- ✓ Peak blood levels of IgG can be obtained within 2 days of intramuscular injection.

The intravenous immune globulin preparation includes alcohol precipitation, fractionation using polyethylene glycol (PEG) or ion exchangers, acidification of the preparation to pH 4.0 - 4.5, treatment with pepsin or trypsin and finally stabilization of the preparation using maltose or glycine to prevent aggregation of globulins.

The preparation should be stored under normal refrigeration between 2°C to 8°C.

Active & Passive Immunizations Vaccine Preparation, Standardization & Storage

VACCINES

Vaccine is an antigenic preparation which is used to attain immunity or resistance against infectious microorganisms. It contains an inactivated germ or a weakened (attenuated) form of live germ or a small part of the germ that acts as antigen to stimulate the immune response.

An ideal vaccine should be able to elicit the following:

1. Activate the antigen presenting cells to initiate antigen processing and produce interleukins.
2. Activate T-lymphocytes and B-lymphocytes to produce huge number of memory cells.
3. Produce T-helper cells and cytotoxic T-cells.
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Types of vaccines: Vaccines are mainly of any of the following types.

1. Killed whole organism
2. Attenuated organism
3. Toxoids
4. Surface molecules
5. Recombinant vector vaccines
6. DNA vaccines

1. **Killed whole organism:** The killed (inactivated) vaccines can stimulate a protective immune response without causing infection. The entire organism is killed or inactivated with chemicals such as formaldehyde to make it harmless.

   Eg: typhoid vaccine, inactivated polio vaccine (IPV), inactivated influenza vaccine, etc.

2. **Attenuated organism:** In this type of vaccine, the organisms are weakened or attenuated by growing them over and over again (also referred as passing) under nourishing conditions to reduce their pathogenicity or disease causing ability.

   Eg: MMR (measles, mumps and rubella) vaccine, oral polio vaccine (OPV), varicella (chicken pox) vaccine, BCG (bacillus calmette Guerin) vaccine.

3. **Toxoids:** These are proteinaceous toxins liberated by the bacteria which are rendered harmless by treating with heat or with formaldehyde to denature the protein. It still retains the capacity to stimulate the formation of tocin antibodies or antitoxins.

Eg: diphtheria toxoid vaccine, tetanus toxoid vaccine.

4. **Surface molecules:** When antibodies bind to the surface of invading pathogen, the destruction of pathogen is triggered, thus protecting the host. Some vaccines contain purified surface molecules that stimulate the production of antibodies. These are alos referred to as component vaccines.

   Eg:

   a. The *influenza* vaccine contains purified surface molecules, i.e. haemagglutinins or inactivated virus particles fro mte three major strains A, B and C.

   b. The hepatitis B vaccine is a recombinant antigen vaccine produced by using recombinant DNA technology. The gene encoding the protein of hepatitis B virus surface antigen or HBsAg can e expressed in yeast cells or *E.coli* and growing them in large fermenters. The cells are cultruredd which then undergo disruption to release the recombinant HBsAg, that is purified by conventional biochemical procedures.

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   d. The vaccines for *Strepococcus pneumniae* and *Neisseria meningitides* consists of purified capsular polysaccharides.

5. **Recombinant vector vaccines:** Attenuated viruses or bacteria serve as a carrier to insert genes coding for antigens of virulent pathogens. The vaccinia virus with genome of 200 genes is used as a vector. The procedure is as follows:

   The plasmid is first cleaved adjacent to the vaccinia promoter using a restriction enzyme where the DNA encoding the antigen from pathogen is inserted. The plasmid is then ligated. The vaccinia virus and recombinant plasmids are allowed to grow in tissue culture cells. Incubation of cells results in insertion of antigen gene and promoter into the vaccinia virus by hologous recombination. Now, the genetically engineered virus can produce high levels of inserted DNA for the antigens.

6. **DNA vaccines:** The plsmid DNA encoding antigen from pathogen is inserted directly into the muscle cells. The inserted DNA induces the production of antigen molecules, which when displayed on the cell surface stimulate both humoral and cell mediated immunity.

   Merits:

   - ✓ A single injection of DNA vaccine is enough to provide immunity for a long duration.
   - ✓ The genes encoding the antigens form several pathogens can be combined and given as a single injection, thus protecting the animal from multiple diseases.
   - ✓ Special care need not be taken while handling DNA vaccine as these are considered to be extremely stable.

1. Poliomyelitis vaccine

Poliomyelitis is a dreaded disease caused by poliovirus that may attack CNA and cause paralysis. Three strains of poliomyelitis virus with distinct antigenic properties are type I, II and III. The general methodology for the preparation of polio vaccine is as below. The three strains are cultured separately in suspended or fixed cell cultures of Rhesus monkey kidney tissue that have been examined thoroughly for any disease both before and after the death of the animal. Poliomyelitis vaccine is of two types.

1. **Inactivated (killed) vaccine (Salk type polio vaccine):**
   This type of vaccine was developed by an American virologist, Dr. Jonas Salk. The viral suspension obtained in the tissue culture is tested for the presence of correct strain of poliomyelitis virus. To make it free from contaminating agents like viruses, bacteria and fungi, this suspension is passed through a series of filters with decreasing thickness.
   This is followed by inactivation of the viral particles using 0.01%formaldehyde solution at pH, temperature and with magnetic stirring for 10-13 days to ensure that no active virus remains.
   The univalent vaccines are mixed to form the trivalent product. This again is tested for the presence of contaminants, if any. This is followed by the addition of sodium metabisulphite to neutralize the formaldehyde and thiomersal to form products that are toxic to the virus.
2. **Attenuated (avirulent) vaccine (Sabin type oral vaccine)**
   This type of vaccine was developed by Dr. Albert Sabin, an American scientist. The methodology for developing is similar to the Salk type vaccine with the exception that-
   1. The strains are developed by repeated passages in monkey kidney cells.
   2. Inactivation of viral particles is not involved.
   3. Through testing for contaminants as well as the presence of virulent forms of poliomyelitis virus is essential.

Storage: The vaccine must be stored at -200C or below under frozen state for long term storage upto 2 years. When the vaccine is thawed (liquid form), it must be kept in refrigerator at 20C to 80C. Vaccine if stored at 20C to 80C shall remain good for only 4-6 months.

2. Tetanus

Tetanus vaccine, also known as **tetanus toxoid** (**TT**), is an inactive vaccine or exotoxins released by *Clostridium tetani* used to prevent tetanus (lock jaw) caused by Clostridium tetani.

Clostridium tetani is a spore-forming anaerobic bacillus. Spores are present in the environment, particularly in the soil of warm and moist areas, and may be carried in the intestinal tracts of humans and animals.

Under favourable anaerobic conditions, such as in dirty, necrotic wounds, this ubiquitous bacillus may produce an extremely potent neurotoxin which causes muscular stiffness and spasm. Characteristically, early spasms of the facial muscles ("lockjaw") are followed by spasm of the back muscles and sudden, generalized tonic seizures and causes disease.

Preparation of vaccine:

Immunity to tetanus is antibody-mediated and depends upon the ability of antitoxin antibodies to neutralize tetanus toxin. Recovery from clinical tetanus does not result in protection against future disease, and immunity can be obtained only by active or passive immunization such as vaccination, immunoglobulin therapy, or transfer of maternal antibodies through the placenta.

Tetanus vaccine is prepared by the exotoxins released from anaerobic bacteria, *Clostridium tetani* cultured in a suitable growth media. The powerful nerve toxin produced by this bacterium causes tetanus.
The exotoxins converted to a taxoid by treating with formaldehyde solution. This is followed by the addition of antimicrobial preservative to the final product.

Toxigenic strains of *C. tetani* are grown in liquid media, the toxin is purified, and then inactivated by treatment with formaldehyde to produce the toxoid antigen.

After purification and sterilization, tetanus toxoid is formulated with aluminum or calcium salts and administered by intramuscular injection.

Tetanus toxoid vaccine is available as a single antigen vaccine (TT), in combination with diphtheria toxoid in infant and adult doses (DT and Td), and in combination with diphtheria and whole-cell or acellular pertussis (DTP). DTP-containing multi-antigen vaccines (with Hep B, Hib, or IPV) are increasingly in use in national immunization campaigns.

Standardization and storage:

The prepared vaccines were checked for the content of exotoxins per ml and it should meet the standards given by WHO and are stored by freeze drying.

During childhood five doses are recommended, with a sixth given during adolescence. Additional doses every 10 years are recommended. After three doses almost everyone is initially immune.

In those who are not up to date on their tetanus immunization a booster should be given within 48 hours of an injury. In those with high risk injuries who are not fully immunized tetanus antitoxin may also be recommended.

3. Cholera

Cholera is a serious infection of the intestine caused by the bacterium, *Vibrio cholerae.* Its symptoms include severe vomiting and diarrhoea leading to dehydration. Vaccination against this disease is effective for 8-9 months.

Method of preparation: The vaccine for cholera is a type of killed bacterial vaccine.

Cholera vaccine is prepared form two classical types of *Vibrio cholera,* Inaba and Ogawa.

The bacterial cells are cleared of contaminants (if any), inoculated in a culture medium (solid or liquid) and incubated for about 3 days in appropriate conditions.

When the cells are grown in a solid culture medium, the culture is washed with saline to remove the cells and then centrifuged to remove agar that would have got detached during the washing process.

When the cells are grown in liquid culture media, following centrifugation, the cells settle at the bottom of the centrifuge tube. The cells are washed and centrifuged twice or thrice to remove the broth constituents.

Then the organisms are killed by heat treatment or by the addition of bactericide such as phenol or formaledehyde.

The bacterial cells per ml are then estimated by either Helber cells, brown tubes or photoelectric nephlometer.

Storage: A suitable preservative is added to the final preparation and stored at a temperature between 2°C and 8°C.

4. BCG Vaccine

BCG vaccine is a type of attenuated bacterial vaccine and is used as a preventive vaccine against tuberculosis in humans.

Preparation of BCG vaccine:

This vaccine consists of attenuated strains of *Mycobacterium bovis.* The basic principle underlying the use of BCG vaccine is to develop resistance in the subject by producing a tuberculosis infection. However, being attenuated, the mycobacteria is only capable of giving rise to local lesions and lymphadenopathy which tends to regress with time.

BCG Vaccine for percutaneous use, is an attenuated, live culture preparation of the Bacillus of Calmette and Guerin (BCG) strain of Mycobacterium bovis.

The medium in which BCG organism is grown for preparation of the freeze-dried cake is composed of the following ingredients: glycerin, asparagine, citric acid, potassium phosphate, magnesium sulfate, and iron ammonium citrate.

The final preparation prior to freeze drying also contains lactose.

Standardization and storage:

The freeze-dried BCG preparation is delivered in vials, each containing 1 to 8 x 108 colony forming units (CFU) of BCG which is equivalent to approximately 50 mg wet weight. Determination of *in-vitro* potency is achieved through colony counts derived from a serial dilution assay.

Intradermal guinea pig testing is also used as an indirect measure of potency. Reconstitution requires addition of Sterile Water for Injection, U.S.P. at 4–25°C (39-77°F). For an adult dosage, 1mL of Sterile Water for Injection, U.S.P., should be added to one vial of vaccine. For a pediatric dosage, 2mL of Sterile Water for Injection, U.S.P., should be added to one vial of vaccine

No preservatives have been added.

Generally infants less than 6 months of age are administered the BCG vaccine. Usually, prior to administration, the individual is subjected to tuberculin testing. However, such testing is not necessary in infants.

0.1 ml of BCG vaccine is administered intradermally into the deltoid region only when two consecutive pre testings with purified protein derivative (PPD) gives negative results. At the site of injection, a papule develops, which within 1-1½ month transform into an ulcer. This ulcer may eventually undergo healing but the papule is evident for about a year. After vaccination, the subject tests positive for tuberculin skin test which indicates that the BCG vaccine has conferred immunity.

5. Typhus vaccine

Typhus (spotted fever) is a type of infection caused by rickettsiae, a non motile spherical or rod shaped bacteria that cannot reproduce outside of their hosts. It is characterized by headache, skin rash, prolonged high fever and delirium.

Preparation of vaccine: The typhus vaccine is prepared in the following manner.

- ✓ The fertile eggs are first incubated for one week.
- ✓ The virulent forms of rickettsiae are injected into the yolk sac of fertile eggs.
- ✓ After about 13 days of injection, the yolk sacs from the eggs are removed under sterile conditions and homogneized to free the rickettsiae.
- ✓ The homogenized suspension is treated with 0.2 to 0.5% w/w of yolk sac tissue.
- ✓ Further purification is done by treating with ether or trichlorotrifluoroethane.
- ✓ The aqueous layer of the suspension is collected and treated with a bactericide, thiomersal.
- ✓ The resulting preparation is collected in sterile containers under aseptic conditions and sealed properly.

6. Small pox vaccine

Small pox is caused by Variola virus. There are two forms of the virus, Variola major and Variola minor.

Preparation of vaccine:

Unlike other vaccines, small pox vaccine is made from virus which is not responsible for causing small pox.

The vaccine is made from a virus called Vaccinia, which is a pox virus, similar to smallpox, but less harmful. It protects people from smallpox by helping their bodies develop immunity to smallpox.

The smallpox vaccine contains live Vaccinia virus, not a killed or weakened virus like many other vaccines.

Traditional methods of preparing small pox vaccine entail passage of Vaccinia virus through living mammalian hosts which have difficulty in obtaining free of bacterial contaminants.

The newer methods include the development of vaccine in chick embryos. A 12 day old chick embryo should be decapitated, eviscerated and cut into pieces.

Then these pieces are washed in phosphate buffered saline and digested with Trypsin. Then the cells are washed free of trypsin by centrifugation process.

The embryonic cells seeded with vaccinia virus are harvestsed and cultured in suitable medium which mainly include earle's saline, hartley's digest broth, native horse serum, L-cysteine hydrochloride, lactalbumen hydrolysate, penicillin, streptomycin etc.

The cells are to be broken by homogenization at $4^{o}C$ to release virus for a shorter duration at high rpm.

Then the amount of virus per ml has to be estimated.

For most people with healthy immune systems, live virus vaccines are effective and safe. Sometimes a person getting a live virus vaccine experiences mild symptoms such as rash, fever, and head and body aches. In certain groups of people, complications from the vaccinia virus can be severe.

Other live virus vaccines currently used include measles, mumps, rubella, and chickenpox.

Standardization and storage:

After its preparation, it was standardized according to the guidelines given by WHO. The effective amount of virus per ml is calculated and stored in ampoules under freeze drying. Smallpox vaccination can protect the host from smallpox for about 3 to 5 years. After that time, its ability to protect you decreases. For long-term protection, a booster vaccination is needed.

The vaccine has been effective in preventing smallpox infection in 95% of those vaccinated. In addition, the vaccine was proven to prevent or substantially lessen infection when given within a few days after a person was exposed to the Variola virus.

7. Immunizing sera

Immune globulins from human serum have been in use for many years to protect against the emergence of diseases. Immunologists have devised a method for the separation of γ-globulin fractions from serum by precipitation with cold ethanol. This method was called “Cohn fractionation”.

The plasma or serum is collected from healthy donors, pooled and used to prepare immune serum globulin (ISG) or human normal immunoglobulin (HNI). If the donors have recovered from a specific infection, or have been immunized against the disease with large doses of Toxoids, then it is designated accordingly.

Eg: tetanus immune globulin, hepatitis B immune globulin, Varicella Zoster immune, rabies immune globulin, etc.

For instance, tetanus immune globulin a sterile solution of globulins obtained from the plasma of adults hyperimmunized with tetanus toxoid. It is effective against patients who suffer from wounds contaminated with *Clostidium tetani.*

Following the serum collection from donors, the blood cells are removed and returned to the donor. This process is called plasmapheresis. The portion of the serum containing antibody globulins is precipitated by cold ethanol.

The preparation should have the following characteristics.

- ✓ It is free from infectious agents.
- ✓ It is highly concentrated.
- ✓ The preparation is stable for many years.
- ✓ Peak blood levels of IgG can be obtained within 2 days of intramuscular injection.

The intravenous immune globulin preparation includes alcohol precipitation, fractionation using polyethylene glycol (PEG) or ion exchangers, acidification of the preparation to pH 4.0 - 4.5, treatment with pepsin or trypsin and finally stabilization of the preparation using maltose or glycine to prevent aggregation of globulins.

The preparation should be stored under normal refrigeration between 2°C to 8°C.