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MEDICAL MICROBIOLOGY AND IMMUNOLOGY

**The educational-methodical manual
on sections «General microbiology»
and «Theoretical and applied medical immunology»
for 2nd year students of Faculty of general medicine
for overseas students**

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Предназначено для студентов медицинских вузов, обучающихся на английском языке. Пособие состоит из двух разделов, посвящено изучению общей микробиологии и иммунологии, рассчитано на 51 час лабораторных занятий, из них 11 лабораторных занятий (33 часа) по общей микробиологии и 6 лабораторных занятий (18 часов) по теоретической и прикладной медицинской иммунологии.

Учебно-методическое пособие соответствует требованиям высшей школы.

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INTRODUCTION IN MICROBIOLOGY

World of the microorganisms

The microbial world is a biological realm composed of **microorganisms** and **viruses**. **Microbiology** is the branch of biological sciences concerned with the study of these **microbes**. The term **organism** is a descriptive term that implies cellular life. Hence, **microorganisms** are a type of cellular life that is microscopic in size.

Microorganisms are unicellular organisms, too small to be seen with the naked eye. Among all forms of life on the earth, microorganisms predominate in numbers of species and in biomass, but their occurrence is generally unappreciated because of their small size and the need for a microscope to see individual cells. Although a light microscope is generally required to visualize a single microbial cell, microbial colonies and communities can be readily observed in nature.

There are five major groups of microorganisms: **Archaebacteria, Bacteria, Algae, Protozoa**, and **Fungi.** Based on differences in cellular organization the kingdom Protists has been divided into two groups **prokaryotes** and **eukaryotes**. Archaebacteria, Bacteria and blue green algae are prokaryotes. Other algae, protozoa, fungi and slime moulds are eukaryotes (see figure 1).

Archaea are a group of unicellular prokaryotic cells that sometimes produce methane during their metabolism, and which often live in *extreme environments* such as high temperature, low pH or high salt concentrations. They are specifically adapted to these conditions by means of special types of membranes and metabolism.

Bacteria are also unicellular prokaryotic organisms. They have a unique type of cell wall and cell membrane that distinguishes them from Archaea. Bacteria live everywhere that life exists on earth except the most extreme environments, including in associations with animals and plants. Most are beneficial, but some cause disease.

Algae are plant-like, photosynthetic, eukaryotic organisms that live wherever there is light and moisture. They convert carbon dioxide to organic material and produce oxygen during photosynthesis, the same as plants.

Protozoa are animal-like, no photosynthetic eukaryotes common in moist environments, including the intestinal tracts of animals. Most protozoa are motile because they are predatory on other microbes and have to catch and ingest their food. A few of them cause some important diseases, such as malaria and sleeping sickness.

Fungi The kingdom includes mushrooms, molds and yeasts. Molds and yeasts are considered microbes. They are filamentous or unicellular eukaryotes, generally non-motile, that absorb their nutrients directly from the environment. Molds live mainly in the soil and are responsible for the decomposition (biodegradation) of organic material. They are also an important cause of plant disease and food spoilage. Although molds grow by filament formation, and form macroscopic colonies, their reproductive structures (spores) are microscopic and can give rise to the complete organism.

Viruses are not cells at all (and therefore, are not really organisms) and are so small that they cannot be viewed by a conventional light microscope.

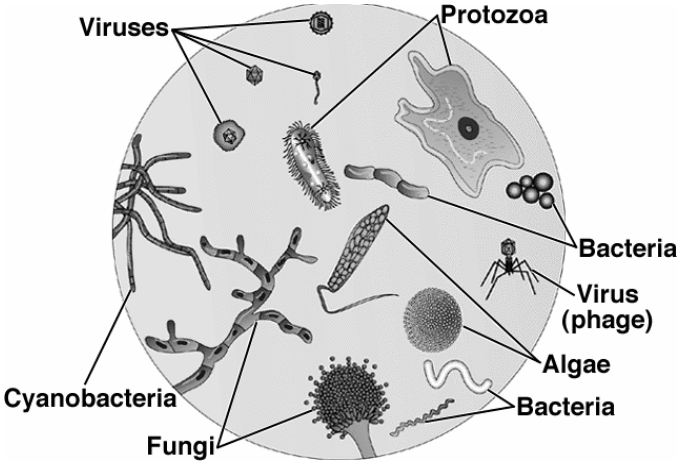


Figure 1 — World of microbes

The study of viruses developed within the field of microbiology, and therefore virology is considered a branch of microbiology. Viruses may be considered as microbes, but they are not microorganisms. Viruses are made up of nucleic acid (DNA or RNA) and protein and have some of the characteristics of life. But they lack ribosomes (for their own protein synthesis), membranes, and the means to generate energy, which are properties of cells.

Viruses are considered **obligate intracellular parasites** because they can only replicate in association with a host cell which they infect. Although viruses are too small to be seen by conventional light microscopy, they can be visualized and photographed by means of electron microscopy.

Branches of microbiology

Microbiology is the study of *microorganisms*, which are unicellular or cell-cluster microscopic organisms. This includes **eukaryotes** such as *fungi* and *protists*, and **prokaryotes** such as *bacteria* and *certain algaes*. Viruses, though not strictly classed as living organisms, are also studied. Microbiology is a broad term which includes many branches like Bacteriology, Virology, Mycology, Parasitology and others. A person who specialize in the area of Microbiology is called as “Microbiologist”

Although much is now known in the field of microbiology, advances are being made regularly. The most common estimates suggest that we have studied only about 1% of all of the microbes in any given environment. Thus, despite the fact that over three hundred years have passed since the discovery of microbes, the field of microbiology is clearly in its infancy relative to other biological disciplines such as zoology, botany and entomology.

Subject of microbiology is studying of microorganisms biological properties:

- Morphology
- Physiology
- Systematics
- Genetics
- Ecology (relationship of the microbes and other forms of life with environment).

Classification of microbiological sciences according to the object of research:

1. General microbiology (study of systematics, structural organization, chemical composition, cultivation and genetics of microorganisms).

2. Individual microbiological sciences: bacteriology (study of the prokaryotes), mycology (study of the fungi), protozoology (study of the multicellular parasites), virology (study of the viruses).

Microbiology is both a basic and an applied science. It is not a single subject. It has many areas of specialization.

Classification of microbiological sciences according to applied subjects of investigations:

- Medical and public health microbiology
- Sanitary (food) microbiology
- Veterinary and agricultural microbiology
- Industrial microbiology
- Soil, sea, space microbiology.

Industrial microbiology — is applying of microorganisms in national economy (production of antibiotics, enzymes, aminoacids and other biologically active substances and food products).

Medical microbiology deals with the causative agents of infectious diseases of humans, their reactions to them and the methods of protection against such diseases. Also medical microbiology is involved in finding ways of identifying, preventing and treating bacterial diseases such as tuberculosis and meningitis, and viral diseases ranging from flu to AIDS. Microbiologists in hospital laboratories deal with samples from patients, isolating and identifying the microbes that cause illness, and giving advice on appropriate treatment. They also try to prevent patients from acquiring hospital-acquired (nosocomial) infections and they attempt to trace and eliminate any infections which may occur.

Like hospital microbiologists, **public health microbiologists** isolate and identify pathogens. Their records of cases and outbreaks of microbial infections are analyzed to provide a continuous picture of the progress of infections. If there is risk of an epidemic, they suggest preventive measures, such as quarantine or mass immunization programs.

Medical microbiologists that work in research institutes and universities study topics such as how diseases develop, or the interactions between pathogenic microbes and host cells. Pharmaceutical companies and other industrial research and development agencies may employ medical microbiologists to work on the development of medicines and vaccines.

Why should a future doctor study microbiology, immunology and virology?

At first, in order to know about a nature of the infection diseases and which of microbes can cause the infection diseases. At second, for training of modern methods of diagnostics, effective ways of prevention and treatment of the infection diseases. Studying of immunology gives an opportunity to know the natural mechanisms of a self-defense against of the infection diseases reacted in our organism.

Food microbiology is concerned with microbial production of foods and with food safety. Microbial fermentations have been used for centuries to produce foods such as cheese and yogurt and alcoholic beverages like wine and beer. Microbiologists are involved at all stages of modern food and drink manufacturing processes.

Safety and hygiene are also the concern of microbiologists in the foods industry. As we store food for longer periods, it becomes more difficult to prevent spoilage by microbes. Some spoilage just makes food look unattractive, but food poisoning may be caused by microbial pathogens such as *Salmonella* or *Staphylococcus* growing on food. Microbiologists are employed in quality control to ensure that products are safe and in basic research into food hygiene and preservation.

Microbiologists in research institutes study the ecology of microbes in soil, fresh water, the sea, and other habitats. Microbial activities can be used to avoid or minimize environmental pollution. Factory wastes are treated with suitable cultures or enzymes produced from bacteria. Microbial processes are being developed to clean up pollution — a process known as **bioremediation**.

The main stages of development of microbiology, immunology and virology

Heuristic period (before of a microscope invention): Fracastorius of Verona (1596) proposed a contagium vivum as a possible cause of infection disease.

Descriptive period (the end of XVII — mid. of XIX century): Antony van Leeuwenhoek described Protists and the main shapes of Bacteria for the first time.

Physiological period (mid. of XIX — beginning of XX century): The development of microbiology as a scientific discipline dates from **Louis Pasteur** (1822–1895).

Scientific contributions of L. Pasteur:

- Discovery of the pathogenic microorganisms (*Staphylococcus, Pneumococcus*).
- Development of active (weakened) vaccines for preventive maintenance.
- Microbial nature of fermentation and “disease” of wine and beer.
- Methods of dry heat sterilization and pasteurization.

Scientific contributions of R. Koch:

- Discovery of the pathogenic microorganisms (*anthrax and tuberculosis rod*).
- Development of basic principles of identification of the pathogenic microbes causing disease.

KOCH'S POSTULATES consists of the following 4 steps:

1. To isolate the suspected agent from a disease victim.
2. To grow the agent in pure culture.
3. To infect a healthy host and show that the organism produces the CLASSICAL CLINICAL DISEASE.
4. To isolate the "same" organism from the new victim.

Other Koch’s discoveries:

- Solid agar media for cultivation of microorganisms
- Aniline dyes
- Immersion objective for light microscopy
- Microphotography
- Sterilization by steam.

Immunological period (beginning — middle of XX century): Discovery of immune response. **Metchnikoff** (1883) discovered the phenomenon of phagocytosis and proposed the phagocytic response as the prime defense against the microbial invasion. This led to the cellular theory of immunity. Ehrlich discovered humoral theory of immunity and development of chemotherapy of infectious diseases. Becoming of science immunology.

Virological period (1892): Becoming of science virology (**D.I. Ivanovsky**). Investigation of viruses and the disease caused by them was rendered difficult as

viruses could not be seen under the light microscope or grown in culture media. The existence of such ultramicroscopic microbes was proved when Ivanovsky reproduced mosaic disease in the tobacco plants from which all bacteria had been removed by passage through filters. The first human disease proved to have a viral etiology was yellow fever.

Modern period (mid. of XX century): **Fleming** (1929) made the accidental discovery that the fungus Penicillin produces a substance which destroys staphylococci. This was the beginning of the antibiotic era. Other similar antibiotics were discovered in rapid succession. But soon the development of drug resistance in bacteria presented serious difficulties.

The main modern achievements:

- Decoding of molecular structure of majority bacteria and viruses.
- Discovering of the new forms of life (infectional proteins — **prions** and infectional RNA — **viroids**).
- Engineering of cultivation methods of animal and plant cells.
- Engineering of essentially new methods for infectional diseases diagnostics (immune enzymatic analysis IEA, polymerase chain reaction PCR, hybridization of nucleic acids).
- Discovering of the new agents of virus and bacterial infections (AIDS).

Microbiological methods of research

- **Microscopical or bacterioscopical method** (with using of devices for microscopy).
- **Microbiological method** (isolation of a pure culture of microbes and it identification).
- **Biological (experimental) method** (contamination of the animals and studying of the effects).
- **Immunological (immunobiological) method**:
  - Serological tests.
  - Skin testing (revealing of a specific hypersensitivity — allergy).
  - Methods of estimation of immune status.
- **Molecular-genetic method** (polymerase chain reaction and other amplification procedures coupled with nucleic acid probes carrying specific DNA or RNA base sequences are now widely used for identifying microbes).

Methods of microscopy

The morphological study of bacteria requires the use of microscope. The following types of microscopes are employed now.

Electron microscopy: In the electron microscope, a beam of electrons is used instead of the beam of light. The object which is held in the path of the beam scatters the electrons and produces an image which is focused on a fluorescent screen. Application in microbiology – study of viruses and study of ultra structure of microbial cell.

Light microscopy: There are many types — basic light, immersion, dark field, phase contrast and luminescent (fluorescent).

The **resolving power** of the light microscope is the distance that must separate two points sources of light if they are to be seen as two distinct images. The useful magnification of a microscope is the magnification that makes visible the smallest particles.

Immersion microscopy: Using microscope — is biological light microscope + immersion objective. Effect — immersion oil (placed between glass slide and objective lens) ⇨ eliminates losses of light rays getting in objective lens. Application in microbiology: most frequently uses in bacteriology for microscopic method of research.

Dark field (dark ground) microscopy: Using microscope — is biological light microscope + dark field condenser that illuminate the object with a cone of light without letting any ray of light to fall directly on the objective lens. Only light scattered from the object reaches the objective lens, with the result that the object appears light against a dark background. Application: using for observation of very thin organisms such as spirochetes.

Phase contrast microscopy: Using microscope — is biological light microscope + phase contrast optical design. Effect: the "phase" differences (in refractive index) between bacterial cells and surrounding medium converted into differences in intensity of light. We can see light and dark contrast in the image. Application in microbiology — using for observation of translucent objects, for example, mycoplasmas.

Luminescent microscopy: Using microscope: luminescent microscope. Effect — luminescence of the object in ultraviolet light is registered. Application in microbiology – microscopy of specimen stained with fluorescent dyes (auramine, rhodamine) and evaluation of serological fluorescence reaction.

Principle of the light microscope

It magnifies the image of the object to be visualized through it. Normally the laboratory microscopes provide a magnification of x 40 (scanner), x 100 (low power), x 400 (high power) and x 1000 (oil immersion). Magnification is how much an image is enlarged under a microscope.

Resolving power is the distance that must separate two point sources of light if they are to be seen as two distinct images. The wavelength of the light that illuminate the object limits the resolution. The best compound microscopes can not resolve parts of a specimen that are closer together than about 200 nanometers (0.2 microns). High-quality light microscope generally allow to view the bacterial cells, but it can not view viruses, however, as these tiny objects are smaller than a wavelength of visible light (0.2 microns).

The **imaging system** is composed of **objective**, **prism** and **eyepiece**. The objective magnification of a specimen primarily. The light rays are refracted to 45^0 by prism and get the image on eyepiece plan. Then magnification is secondarily observed by the eyes. The **total magnification** is obtained by multiplying the magnification of the objective with that of the eyepiece.

The **illumination system** is composed of **lamp**, **collector**, **diaphragm** and **condenser**. The light rays from lamp go to pass the collector and illuminate the diaphragm. Then they will be converged by condenser. This system can illuminate the observed specimen on the stage for visual observation (see figure 2).

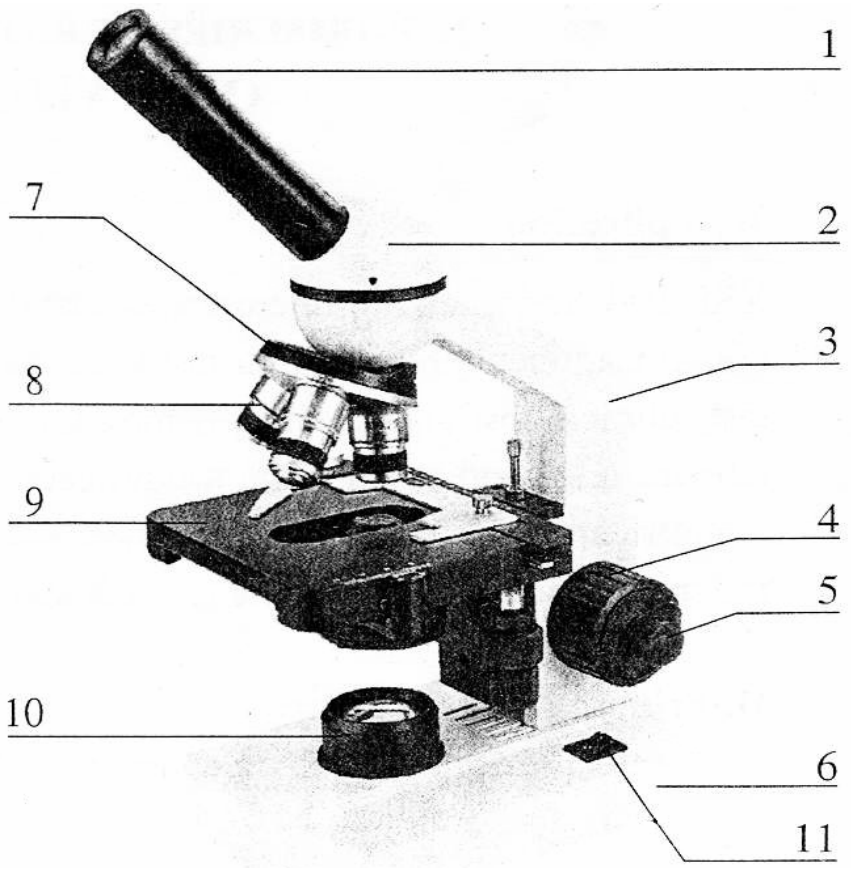


Figure 2 — Biological microscope: 1. Eyepiece; 2. Eyepiece head; 3. Arm; 4. Coarse focus knob; 5. Fine focus knob; 6. Stand; 7. Nosepiece; 8. Objective; 9. Double layer mechanical stage; 10. Collector; 11. Power switch.

Numerical aperture: of the objective is important, depends the amount of light which the lens passes and the detail which it can make visible, on which it is said to resolve.

Oil immersion objectives*:* are used to avoid bending of light beam (with higher magnification). The oil used should have the same optical properties as glass e.g. Cedar Wood oil.

Working of oil immersion objectives*:* A beam of light passing from air into glass is bent and while passing from glass to air it is bent back again. The bending effect and its limitations can be avoided by replacing the air between the specimen and lens with oil which has optical properties similar to that of glass, i.e., immersion oil. When an appropriate oil is used, the light passes in a straight line from glass through the oil and back to glass as through it were passing through glass all the way. Whenever possible the immersion oil recommended by the manufacturer of a microscope should be used.

There are four stage process of using the oil immersion lens:

1. Focus very carefully with the 40x objective over the stained specimen on the slide. (Once focused, do not alter focus for the next three steps!)
2. Rotate turret half way so that the 40x and 100x objectives straddle specimen.
3. Apply a small drop of oil directly on the slide over the specimen.
4. Rotate 100x objective into the immersion oil .

Three important rules attend the use of this lens:

1. Never use an oil immersion lens without the oil.
2. Never get oil on any other lens.
3. Clean up all oil when finished.

Methods of staining

Live bacteria do not show much structural detail under the light microscope due to lack of contrast. So it is customary to use staining techniques to produce colour contrast. Bacteria may be stained in the living state, but this type of staining is used only for special purposes. Long-term methods for staining of bacteria involve drying and fixing smears, procedures that kill them. The following are staining techniques commonly used in bacteriology.

Simple staining: This staining provides the same color to all bacteria

- Staining by ***methylene blue*** or ***aqueous fuchsine***

Aim: revealing of presence of microbes in a pathological material. Study: shape of bacteria and their arrangement in a smear.

Complicated (differential) staining: This staining provides different color to different bacteria.

- **Gram staining** was originally devised by Christian Gram (1884) as a method of staining bacteria in tissues. This method is an important procedure used in the identification of bacteria and frequently is the only method required for studding their morphology. Application — revealing of ***cell wall structure***;
- **Ziehl-Neelsen staining** (**acid fast staining** discovered by Ehrlich) — revealing of ***acid-resistant bacteria*** (Mycobacteria) and revealing of ***spores***;
- **Neisser staining** — revealing of ***volutine storage granules*** and identification of *corynebacteria* according to these granule presence;
- **Burry-Hines staining (negative staining)** — revealing of ***capsules*** when they are seen as clear halos around the bacteria against a black background, using also for revealing ***spirochetes***;
- **Impregnation method (Morozov staining)** — revealing of ***bacterial flagella*** and demonstration of ***spirochetes***, because of these objects too thin to be seen under the light microscope and may be visible if they are thickened be impregnation of silver on the surface;
- **Zdrodovsky staining** — revealing of viruses of chickenpox and smallpox in vesicular lesions; revealing of Rickettsia and Chlamydia;
- **Romanovsky-Giemsa staining** — revealing of Rickettsia and Chlamydia; revealing of Spirochetes after their preliminary differentiation by colour of staining; revealing of parasites.

Bacterioscopical method of microbiological research

Microscopical (bacterioscopical) method of investigation is based on study of morphological and tinctorial properties of microorganisms in investigated material (pathological material, samples from environment, laboratory culture) using different methods of microscopy.

Advantages of the method: simple, widely available, fast, economical.

Disadvantages of the method: not enough sensitive and specific (microbes of different species are similar on the base of their morphology), dangerous.

The aim of microscopical method:

- Determination the disease etiology;
- Morphological identification of microorganisms in investigated material;
- Determination of purity of pure culture.

The types of microscopical preparations employed in the laboratory:

- Usual smear
- Smear-twins from phlegm and pus
- "Hanging drop" or "press drop"
- Thin blood smear
- Thick blood drop
- Smear-stamp.

Steps of bacterioscopical method:

1. Material selection (pus, phlegm, blood, urine, excrements, scrubbing fluid from bronchi and stomach, liquor, cadaveric material, etc.).
2. Material transportation.
3. Slides preparation, there fixation, staining.
4. Microscopy of native or stained slides.
5. Estimation of results.

Success at bacterial staining depends first of all on the preparation of a suitable ***smear*** of the organisms. The first step in preparing a bacteriological smear differs according to the source of the organisms. If the bacteria are growing in a liquid medium (broths, milk, saliva, urine, etc.), one starts by placing one or two dozes of the liquid medium directly on the slide. From solid media such as nutrient agar, blood agar, or some part of the body, one starts by placing one or two doses of water on the slide and then uses a straight inoculating wire to disperse the organisms in the water. Bacteria growing on solid media tend to link to each other and must be dispersed sufficiently by dilution in water; unless this is done, the smear will be too thick.

Preparation of smear

Aseptic transfer (see figure 3) of a culture from one culture vessel to another is successful only if no contaminating microorganisms are introduced in the process.

A transfer may involve the *transport of organisms from an isolated colony on a plate of solid medium to a broth tube*, or *inoculating various media (solid or liquid) from a broth culture for various types of tests*. The general procedures following:

Work area disinfection: The **work area** is first treated with a **disinfectant** to kill any microorganisms that may be present. This step destroys vegetative cells and viruses; endospores, however, are not destroyed in this brief application of disinfectant.

Loops and needles: The transport of organisms will be performed with an inoculating **loop** or **needle**. To sterilize the loop or needle prior to picking up the organisms, heat must be applied with a Bunsen burner flame, rendering them glowing red-hot.

Culture tube flaming: Before inserting the cooled loop or needle into a tube of culture, the tube cap is removed and the mouth of the culture tube flamed. Once the organisms have been removed from the tube, the **tube** mouth must be **flamed** again before returning the cap to the tube.

Liquid medium inoculation: If a tube of liquid medium is to be inoculated, the tube mouth must be flamed before inserting the loop into the tube. To disperse the organisms on the loop, the loop should be twisted back and forth in the medium. If an inoculating needle is used for stabbing a solid medium, the needle is inserted deep into the medium.

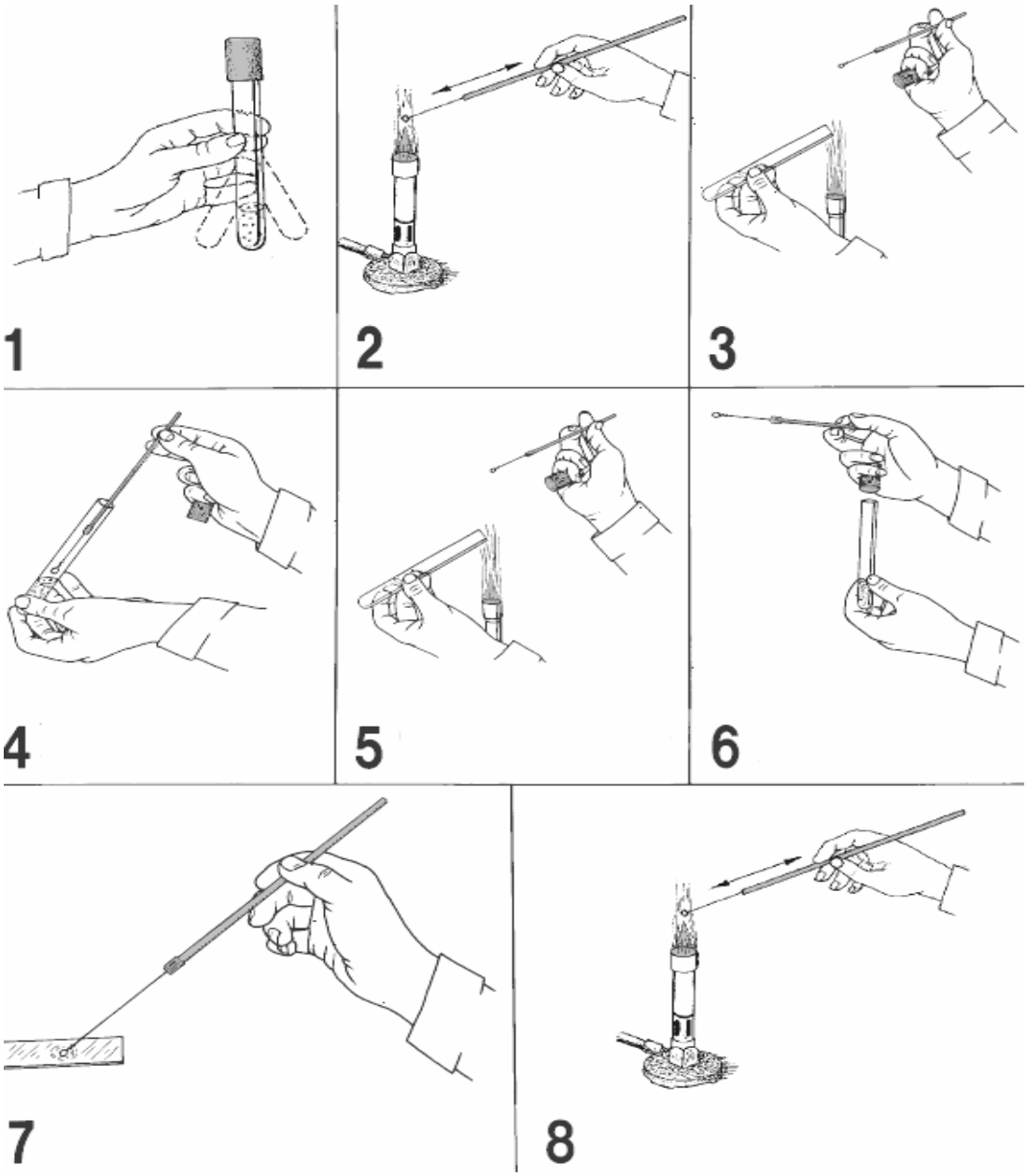


Figure 3 — Aseptic procedure for bacteria removal: 1. Shake the culture tube from side to suspend organisms; 2. Heat the loop and wire to red-hot. Flame the handle slightly also; 3. Remove the cap and flame the neck of the tube. Do not place the cap down on the tube; 4. After allowing the loop to cool for at least 5 seconds, remove the loopful of organisms. Avoid touching the sides of the tube! 5. Flame the mouth of the culture tube again; 6. Return the cap to the tube and place the tube in a test-tube rack; 7. Place the loopful of organisms in the center of the target circle on the slide; 8. Flame the loop again before removing another loopful from the culture or setting the inoculating loop aside.

Final flaming: Once the inoculation is completed, the loop or needle is removed from the tube, flamed as before, and returned to a receptacle. These tools should never be placed on the tabletop. The inoculated tube is also flamed before placing the cap on the tube.

Petri plate inoculation: To inoculate a Petri plate, no heat is applied to the plate and a loop is used for the transfer. When streaking the surface of the medium, the cover should be held diagonally over the plate bottom to prevent air contamination of the medium.

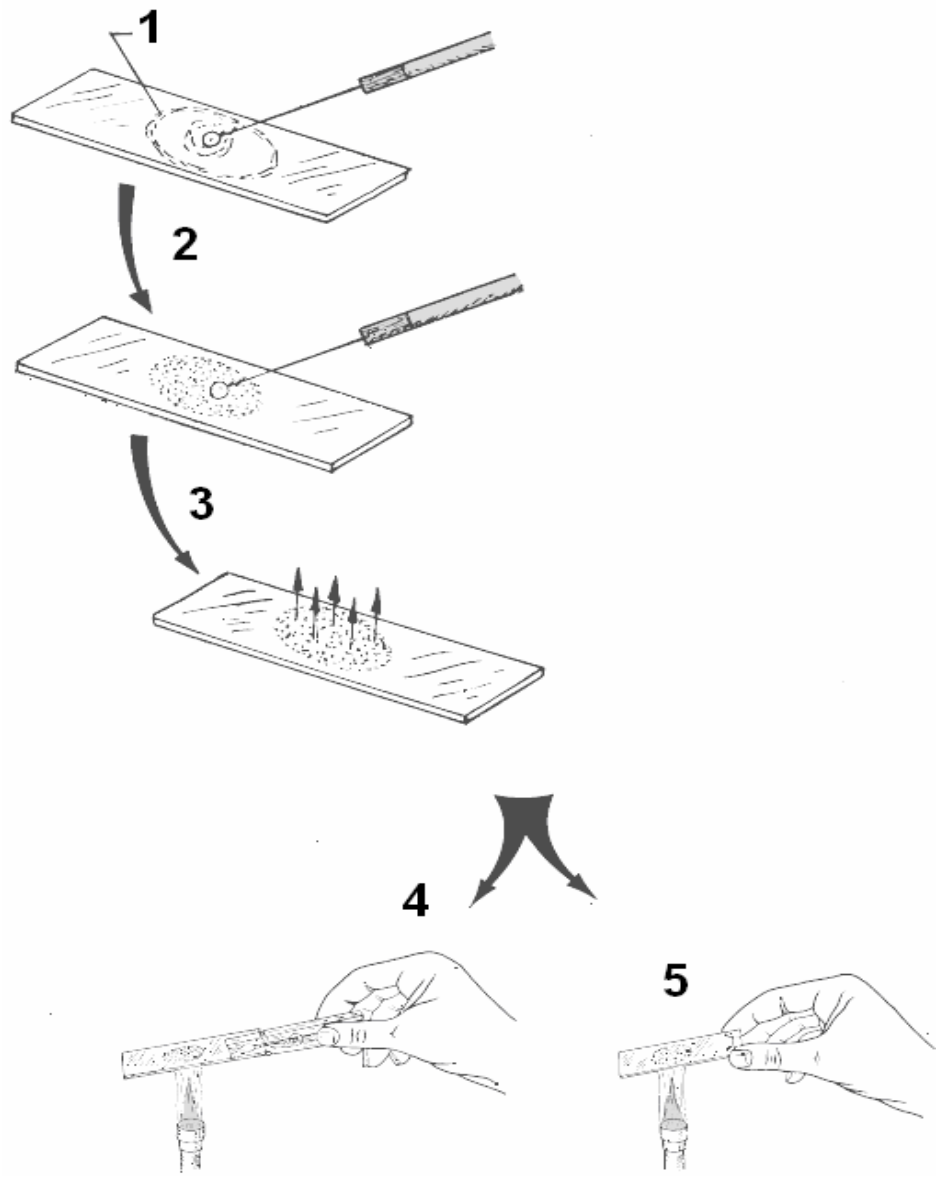


Figure 4 — Procedure for making a bacterial smear: 1. Two loopfuls of physiological solution are placed in center of slide; 2. Very small amount of organisms is dispersedwith inoculating loop in solution; 3. Smear is allowed to dry; 4. Slide is passed through flame several times to heat-kill and fox microorganisms to slide.

Final disinfection: When all work is finished, the work area is treated with disinfectant to ensure that any microorganisms deposited during any of the procedures are eliminated.

Previous operations for inoculation shown in figure 4. Be sure to cool the loop completely before inserting it into a medium! A loop that is too hot will spray the medium and move bacteria into the air. The use of a single stain to color a bacterial organism is commonly referred to as **simple staining** (see figure 5). Some of the most commonly used dyes for simple staining are methylene blue, basic fuchsine, and crystal violet. The staining times for most simple stains are relatively short, usually from 30 seconds to 2 minutes, depending on the affinity of the dye. After a smear has been stained for the required time, it is washed off gently, blotted dry, and examined directly under oil immersion. Such a slide is useful in determining basic morphology and the presence or absence of certain kinds of granules.

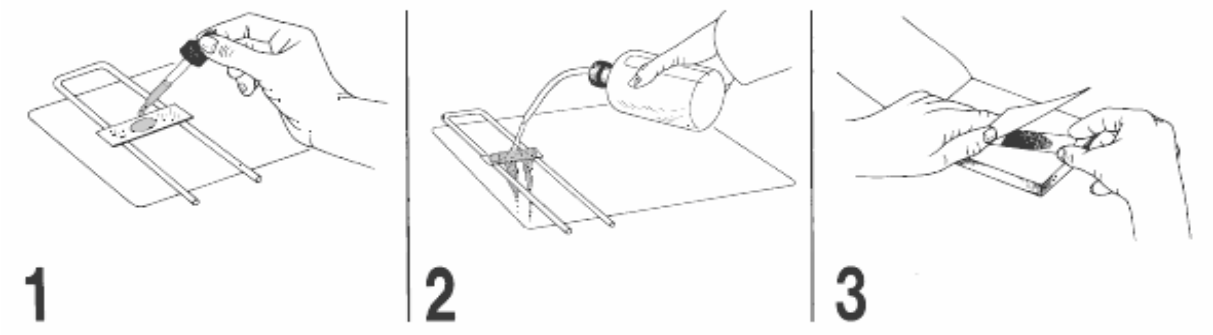


Figure 5 — Procedure for simple staining: **1**. Bacterial smear is stained with methylene blue for one minute; **2.** Stain is brieffy washed off slide with water; **3**. Water drops are carefully blotted off slide with filtering paper.

Taxonomy and classification of the microorganisms

Bacterial taxonomy comprises three components: classification, identification, and nomenclature.

Classification is the orderly arrangement of bacteria into groups. There is nothing inherently scientific about classification, and different groups of scientists may classify the same organisms differently. For example, clinical microbiologists are interested in the serotype, antimicrobial resistance pattern, and toxin and invasiveness factors in *Escherichia coli,* whereas geneticists are concerned with specific mutations and plasmids.

Classification criteria:

1. **Phylogenetical principle** (for large taxons);
2. **Phenotypical principle**
   - Tinctorial properties (ability of microorganisms to stain by different dyes);
   - Cultural properties (characteristic of microbial growth on the liquid or solid medium);
   - Motility;
   - Sporulation (spore shape and it location in the microbial cell);
   - Physiological properties (type of nutrition and respiration);
   - Biochemical properties (ability of microorganisms to ferment the different substrates);
   - Antigenic properties.
3. **Genotypical principle**

Genosystematics — is studying of DNA composition and the most important features of genome (revealing of a genetical alliance among of bacteria according to quantity of identical genes). The ideal means of identifying and classifying bacteria would be to compare each gene sequence in a given strain with the gene sequences for every known species. This cannot be done, but the total DNA of one organism can be compared with that of any other organism by a method called **nucleic acid hybridization or DNA hybridization**. This method can be used to measure the number of DNA sequences that any two organisms have in common and to estimate the percentage of divergence within DNA sequences that are related but not identical. DNA relatedness studies have been done for yeasts, viruses, bacteriophages, and many groups of bacteria. Five factors can be used to determine DNA relatedness: genome size, guanine-plus-cytosine (G+C) content, DNA relatedness under

conditions optimal for DNA reassociation, thermal stability of related DNA sequences, and DNA relatedness under conditions supraoptimal for DNA reassociation.

4. **Mixed principle** (using a great number of attributes).

Hierarchical system of taxons used in bacteriology

- **Kingdom** (Prokaryote)
- **Division**
- **Order.** Name of the taxon is ended with –ales.
- **Family.** A group of related genera**.** Name of the taxon is ended with –ceae.
- **Tribe**
- **Genus (genera).** A genus is a group of related species.
- **Species.** Species is the taxonomical unit in microbiology. A **bacterial species** is a distinct organism with certain characteristic features, or a group of organisms that resemble one another closely in the most important features of their organization.

Species' subdivisions

Below the species level, strains are designated as groups or types on the basis of common serologic or biochemical reactions, phage or bacteriocin sensitivity, pathogenicity, or other characteristics.

Variants (subspecies): morphovariant, biovariant, phagovariant, serovariant, pathovariant, chemovariant.

Morphovariant (**morphovar**) is groups of microorganisms distinguished by morphological properties (size, shape and others).

Biovariant (**biovar**) is groups of microorganisms distinguished by biological features (features of cultivation – nutrients, conditions and others).

Chemovariant (**chemovar**) is groups of microorganisms distinguished by features of the fermentation on the different substrates.

Serovariant (**serohovar**) is groups of microorganisms distinguished by antigenic features (set of superficial antigens of the microbes).

Pathovariant (**pathohovar**) is groups of microorganisms distinguished by pathogenic factors (adaptations of microbes allowed them to carry out a process of victim contamination).

Phagovariant (**phagovar**) is groups of microorganisms distinguished by sensitivity to the antibiotics and bacteriophages (viruses of bacteria).

Hierarchical system of taxons used in virology

- **Kingdom** (Vira)
- **Subkingdom**

DNA-genomic viruses and RNA-genomic viruses.

- **Family**

Name of the taxon is ended with –viridae.

- **Subfamily**

Name of the taxon is ended with –virinae.

- **Species**

Name of the taxon is ended with –virus.

- **Serological variants**

On the basis of antigenic structure.

Identification is the practical use of classification criteria to distinguish certain organisms from others, to verify the authenticity or utility of a strain or to isolate and identify the organism that causes a disease. **Identification of microbe** is determina-

tion of microbe taxonomic position (especially — species of given microbe). It is a main moment of bacteriological diagnostics of the infection diseases. For pathogenic bacteria identification is necessary to study the morphological, tinctorial, cultural, biochemical and antigenic properties.

- **Strain** (microbial culture, isolated from certain source)
- **Clone** (generation having origin from one cell)
- **Pure culture** (population of the same microbes isolated from one colony and grown on the solid nutrient medium).

Phenotypic characteristics useful in classification and identification:

1. Morphologic characteristics

Staining simple tests can indicate the Gram reaction of the organism; whether it is acid-fast; its motility; the arrangement of its flagella; the presence of spores, capsules, and inclusion bodies; and, of course, its shape. Colony characteristics and pigmentation are also quite helpful.

2. Growth characteristics

A primary distinguishing characteristic is whether an organism grows aerobically, anaerobically, facultatively, or microaerobically (i.e., in the presence of a less than atmospheric partial pressure of oxygen). The proper atmospheric conditions are essential for isolating and identifying bacteria. Other important growth assessments include the incubation temperature, pH, nutrients required, and resistance to antibiotics.

3. Antigens and phage susceptibility

Cell wall (O), flagellar (H), and capsular (K) antigens are used to aid in classifying certain organisms at the species level, to serotype strains of medically important species for epidemiologic purposes, or to identify serotypes of public health importance. Serotyping is also sometimes used to distinguish strains of exceptional virulence or public health importance, for example *E coli* (enterotoxigenic, enteroinvasive, enterohemorrhagic, and enteropathogenic serotypes).

Phage typing (determining the susceptibility pattern of an isolate to a set of specific bacteriophages) has been used primarily as an aid in epidemiologic surveillance of diseases caused by *Staphylococcus aureus,* mycobacteria, *V cholerae,* and *S typhi.* Susceptibility to bacteriocins has also been used as an epidemiologic strain marker.

4. Biochemical characteristics

Most bacteria are identified and classified largely on the basis of their reactions in a series of biochemical tests. Some tests are used routinely for many groups of bacteria (oxidase, nitrate reduction, amino acid degrading enzymes, fermentation or utilization of carbohydrates); others are restricted to a single family, genus, or species (coagulase test for staphylococci, pyrrolidonyl arylamidase test for Gram-positive cocci).

Nomenclature (naming) is the means by which the characteristics of a species are defined and communicated among microbiologists. A species name should mean the same thing to all microbiologists, yet some definitions vary in different countries or microbiologic specialty groups. For example, the organism known as *Clostridium perfringens* in the United States is called *Clostridium welchii* in England.

For designation of the species using **binary nomenclature** consisted of generic name and *specific name*. For example, *Neisseria meningitides*.

The modern taxonomy of bacteria

First time issued in 1923 by group of the authors (led by D. Bergy) determinant of bacteria was received the international recognition. Then Berg`s determinant was repeatedly processed and was republished 8 times. The last 9th edition **"Bergey's Manual of Determinative Bacteriology–9"** was published in 1993.

According to the Berg`s determinant the Kingdom Prokaryote is divided into departments distinguished from each other by a structure of a cell wall and the attitude to Gram staining.

There are 4 divisions of Prokaryote:

Division I. **GRACILICUTES** (*Gram-negative eubacteria* which have a thin cell wall).

Division II. **FIRMICUTES** (*Gram-positive eubacteria* which have a thick cell wall).

Division III. **TENERICUTES** (*Eubacteria* which lack a cell wall, Mycoplasma).

Division IV.**MENDOSICUTES** (*Archaebacteria* which have cell wall without peptidoglycan — the main component of eubacterial cell wall).

MORPHOLOGY AND ULTRASTRUCTURE OF BACTERIAL CELL

Procaryotes and eucaryotes

"True" bacteria (which include all bacteria that infect man) are members of one kingdom (the eubacteria, bacteria). In addition, a group of organisms often found in extreme environments form a second kingdom (**archaebacteria** or *Archaea*). Morphologically, the two kingdoms of organisms appear similar, especially in the absence of a nucleus, and thus are classified together as prokaryotes. However, they have major biochemical differences. Most archaea live in environments such as hot sulfur springs where they experience temperatures as high as 80 degrees C and a pH of 2. These are called thermoacidophiles. Others live in methane-containing (**methanogens**) or high salt (**extreme halophiles**) environments.

Eubacteria (with the exception of the genera *Mycoplasma* and *Chlamydia*) possess **peptidoglycan** (synonyms: murein, mucopeptide, cell wall skeleton). Peptidoglycan contains a unique sugar, muramic acid, not found elsewhere in nature. Archaebacteria contain a **pseudomurein** that is different in structure from eubacterial murein. Members of the *Archaea* are not human pathogens and will not be discussed further.

Differences between prokaryotes and eukaryotes

Biologists recognize the existence of two fundamentally different types of cells in the microbial world, called **prokaryotic** and **eukaryotic** cells. Eukaryotic cells have a "true" nucleus (the region of the cell that contains genetic information or DNA) because it is enclosed in a nuclear membrane; prokaryotic cells are said to have a "primitive" nucleus because their DNA is not enclosed within a nuclear membrane. The nuclear region of a prokaryotic cell is sometimes referred to as a **nucleoid**, rather than as a nucleus.

The prokaryotic cell, in contrast to the eukaryotic cell, is not compartmentalized. Nuclear membranes, mitochondria, endoplasmic reticulum, Golgi body, phagosomes and lysosomes are not present. Prokaryotic ribosomes are 70S (S stands for Svedberg unit, a measure of size), whereas eukaryotic ribosomes are larger (80S). Bacterial membranes generally do not contain sterols (e.g. cholesterol).

Morphological features of bacteria

1. **Structural components**:

Obligate (strict) components of bacterial cell: cell wall, plasma membrane, nucleoid, mesosomes and ribosomes. **Facultative components of bacterial cell:** pili, flagella, plasmids, capsules, spores and inclusions.

2. **Shape** and **size** of bacteria;
3. **Tinctorial properties** (e.g. Gram reaction);
4. **Motility** (presence of flagella);
5. **Protective devises** (spores and capsules);
6. **Arrangement of cells** in a smear.

Size and shape of bacteria

Bacteria of medical importance generally measure 0.2–1.5 μm in diameter and about 3–5 μm in length. Depending on their shape (see figure 6), bacteria are classified into several varieties:

1. **COCCI** (from *kokkos* meaning berry) are spherical or oval cells (see figure 6, 7).
2. **BACILLI** (from *bacillus* meaning rod) are rod shaped cells (see figure 8).

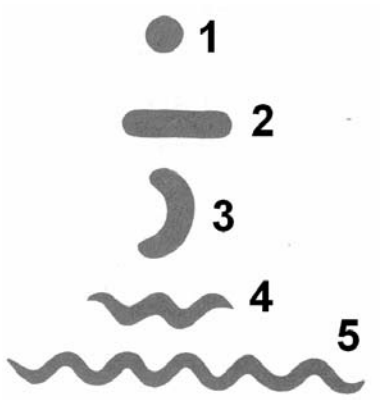


Figure 6 — Shapes of bacteria:
1. Coccus; **2**. Bacillus;
3. Vibrio; **4**. Spirillum;
5. Spirochete.

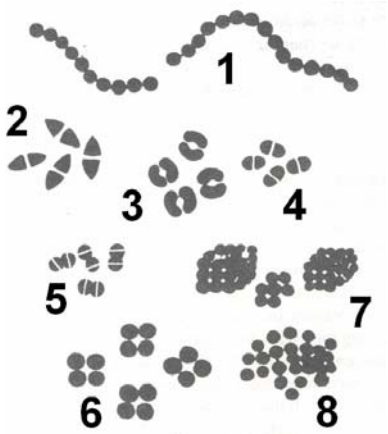


Figure 7 — Arrangement of cocci:
1. Streptococci; **2**. Pneumococci;
3. Gonococci; **4.** Meningococci;
5. N*eisseria catarrhalis*; **6**. Tetrads;
7. Sarcina; **8**. Staphylococci.

3. **SPIRAL FORMS** (see figure 9):
   ◦ VIBRIOS are comma shaped curved rods and derive the name from their characteristic vibratory motility.
   ◦ SPIRILLA are rigid spiral forms.
   ◦ SPIROCHETES are flexuous spiral forms.
4. **ACTINOMYCETES** are branching filamentous bacteria, so called because of resemblance to the radiating rays of the sun when seen in tissue lesions (from *actis* meaning ray and *mykes* meaning fungus). The characteristic shape is due to the presence of a rigid cell wall.
5. **MYCOPLASMAS** are bacteria that have cell wall deficient and hence do not possess a stable morphology. They occur as round or oval bodies and as interlacing filaments. When cell wall synthesis becomes defective bacteria lose their distinctive shape. Such cells are called protoplasts, spheroplasts and L-forms.

Bacteria sometimes show characteristic cellular arrangement or grouping. Thus, cocci may be arranged in pairs (diplococci), chains (streptococci), groups of four (tetrads) or eight (Sarcina), or grape-like clusters (staphylococci).

Some bacilli too may arrange in chain (streptobacilli). Other arranged at angles to each other, presenting Chinese letter pattern (*Corynebacteria*). The type of cellular arrangement is determined by the plane through which binary fission takes place and by the tendency of the daughter cells to remain attached even after division.

Some species of bacteria exhibit great variation in the shape and size. This known as **pleomorphism**. Certain species (e.g. gonococcus) show swollen and aberrant forms in ageing cultures, especially in presence of high salt concentration. There are known as **involution forms**.

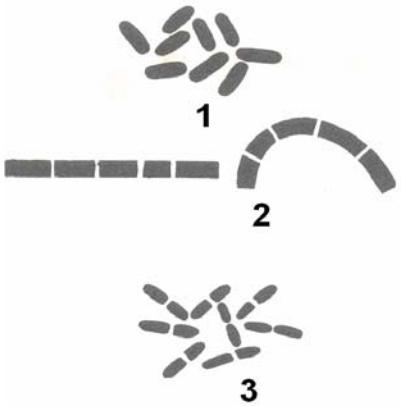
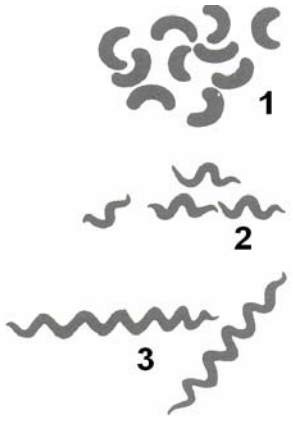


Figure 8 — Arrangement of bacilli:
1. Bacilli in cluster;
2. Bacilli in chain (*B. anthrax*);
3. Diplobacilli (*K. pneumonia*).



Figbre 9 — Arrangements of spiral bacteria:
1. Vibrio;
2. Spirilla;
3. Spirochetes.

The bacterial cell

Procaryotes are unicellular organisms of relatively simple construction, especially if compared to eukaryotes. A prokaryotic cell has five essential structural components: a **genome (DNA)**, **ribosomes**, **cell membrane**, **cell wall**, and some sort of **surface layer** *(capsule)* which may or may not be an inherent part of the wall. The development of the electron microscope, in the 1950s, revealed the distinct anatomical features of bacteria and confirmed the suspicion that they lacked a nuclear membrane.

Structurally, a prokaryotic cell (figure 10 below) has three architectural regions: **appendages** (attachments to the cell surface) in the form of **flagella** and **pili (or fimbriae)**; a **cell envelope** consisting of a capsule, **cell wall** and **plasma membrane**; and a **cytoplasmic region** that contains the cell **genome** (**DNA**) and **ribosomes** and various sorts of **inclusions**.

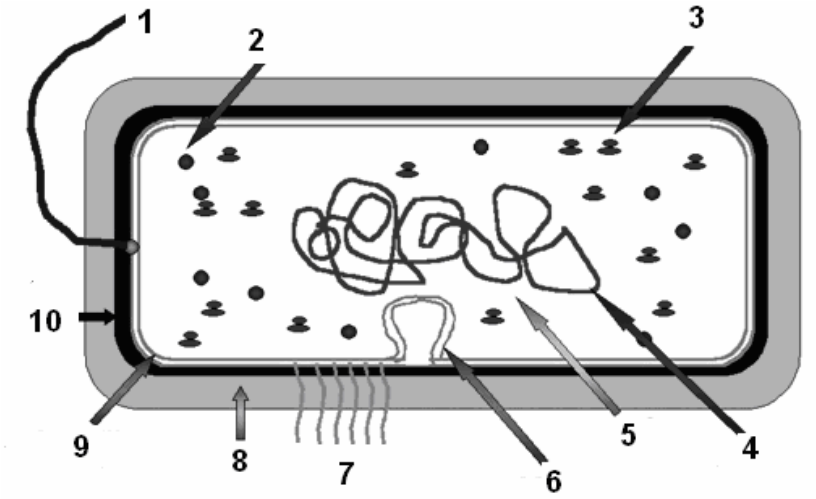


Figure 10 — Drawing of a typical prokaryotic cell:
1. Flagellum; **2.** Inclusions; **3.** Ribosomes; **4**. Nucleoid; **5**. Cytoplasm; **6**. Mesosome; **7**. Pili; **8**. Capsule; **9**. Cytoplasmic membrane; **10.** Cell wall/ outer membrane (if present).

Table 1 — **Characteristics of typical bacterial cell structures**

Structure	Function(s)	Predominant chemical composition
Flagella	Swimming movement	Protein (flagellin)
Pili Sex pili	Mediates DNA transfer during conjugation	Protein
Common pili or fimbriae	Attachment to surfaces; protection against phagotrophic engulfment	Protein
Capsules (includes "slime layers" and glycocalyx)	Attachment to surfaces; protection against phagocytic engulfment, occasionally killing or digestion; reserve of nutrients or protection against desiccation	Usually polysaccharide; occasionally polypeptide
Cell wall Gram-positive bacteria	Prevents osmotic lysis of cell protoplast and confers rigidity and shape on cells	Peptidoglycan (murein) complexed with teichoic acids
Gram-negative bacteria	Peptidoglycan prevents osmotic lysis and confers rigidity and shape; outer membrane is permeability barrier; associated LPS and proteins have various functions	Peptidoglycan (murein) surrounded by phospholipid protein-lipopolysaccharide "outer membrane"
Plasma membrane	Permeability barrier; transport of solutes; energy generation; location of numerous enzyme systems	Phospholipid and protein
Ribosomes	Sites of translation (protein synthesis)	RNA and protein
Inclusions	Often reserves of nutrients; additional specialized functions	Highly variable; carbohydrate, lipid, protein or inorganic
Chromosome	Genetic material of cell	DNA
Plasmid	Extrachromosomal genetic material	DNA

Flagella and bacterial motility

Flagella are filamentous protein structures attached to the cell surface that provide the swimming movement for most motile procaryotes. Prokaryotic flagella are much thinner than eukaryotic flagella, and they lack the typical "9 + 2" arrangement of microtubules. The diameter of a prokaryotic flagellum is about 20 nanometers. The flagellar filament is rotated by a motor apparatus in the plasma membrane allowing the cell to swim in fluid environments. Bacterial flagella are powered by proton motive force (chemiosmotic potential) established on the bacterial membrane, rather than ATP hydrolysis which powers eukaryotic flagella.

The presence or absence of flagella and their number and arrangement are characteristic of different genus of bacteria. So flagellar distribution is a genetically-distinct trait that is occasionally used to characterize or distinguish bacteria. For example, among Gram-negative rods, pseudomonads have polar flagella to distinguish them from enteric bacteria, which have peritrichous flagella.

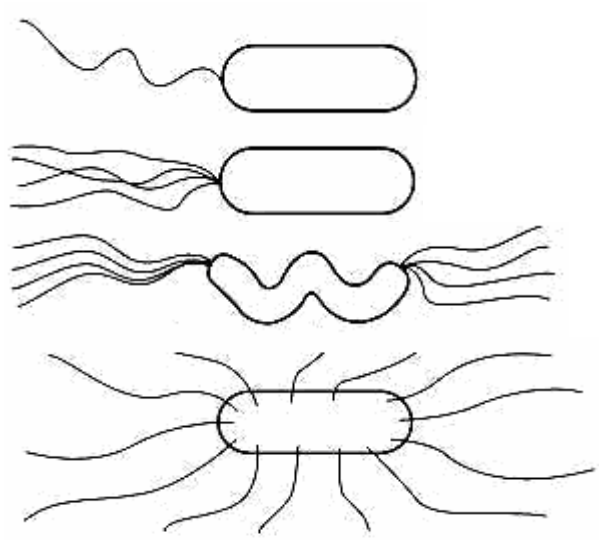
About half of the bacilli and all of the spiral and curved bacteria are motile by means of flagella. Very few cocci are motile, which reflects their adaptation to dry environments and their lack of hydrodynamic design. Flagella may be variously distributed over the surface of bacterial cells in distinguishing patterns, but basically flagella are either **polar** (flagella are situated at one or both end of the cell) or **peritrichous** (flagella are arranged all round the cell).

Bacterial classification according to number and localization of flagella:

1. Monotrichous (polar flagella may be single).

2. **Polytrichous** (many flagella with different distribution):
   - Amphitrichous (flagella at both poles of the bacterial cell);
   - Lophotrichous (tufts of flagella localized at the end of the cell);
   - Peritrichous (flagella are arranged all round the cell).
3. **Atrichous** (lack of a flagella). This is illustrated in figure 11.

The ultrastructure of the flagellum of *E. coli* is illustrated in figure 12. The flagellar apparatus consists of several **distinct proteins**: a system of **rings** embedded in the cell envelope (the **basal body**), a **hook-like structure** near the cell surface, and the **flagellar filament**. The innermost rings, the M and S rings, located in the plasma membrane, comprise the motor apparatus. The outermost rings, the P and L rings, located in the periplasm and the outer membrane respectively, function as bushings to support the rod where it is joined to the hook of the filament on the cell surface. As the M ring turns, powered by an influx of protons, the rotary motion is transferred to the filament which turns to propel the bacterium.



Structure	Flagella type	Example
	Monotrichous	Vibrio cholerae
	Lophotrichous	Bartonella bacili-formis
	Amphitrichous	Spirillum serpens
	Peritrichous	Escherichia coli

Figure 11 — Different arrangements of bacterial flagella

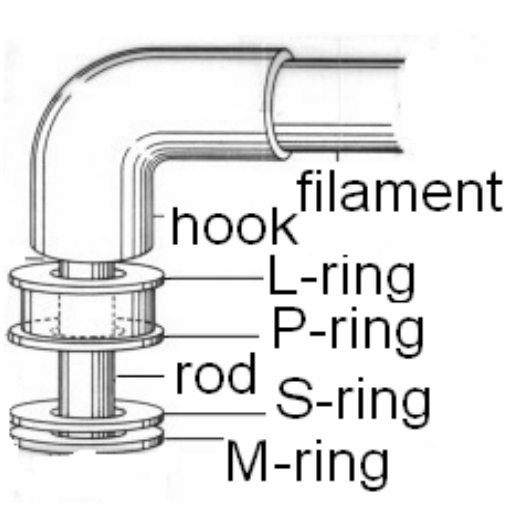


Figure 12 — Structure of flagella

Procaryotes are known to exhibit a variety of types of **tactic behavior**, i.e., the ability to move (swim) in response to environmental stimuli. For example, during **chemotaxis** a bacterium can sense the quality and quantity of certain chemicals in its environment and swim towards them (if they are useful nutri-ents) or away from them (if they are harmful substances). Other types of tactic response in procaryotes include **phototaxis**, **aerotaxis** and **magnetotaxis**. The occurrence of tactic behavior provides evidence for the ecological (survival) advantage of flagella in bacteria and other procaryotes.

Detection of bacterial motility

Since motility is a primary criterion for the diagnosis and identification of bacteria, several techniques have been developed to demonstrate bacterial motility, directly or indirectly.

1. **Flagellar stains (impregnation method)** outline flagella and show their pattern of distribution. If a bacterium possesses flagella, it is presumed to be motile. Flagella may, in some instances, be seen under dark field microscopy or electron microscopy.

2. **Motility test medium** demonstrates if cells can swim in a semisolid medium (spreading type of bacterial growth).

3. **Direct microscopic observation** of living bacteria in a preparation "hanging drop". Active motility has to differentiated from the passive movements of the cells (Brownian movement). Bacterial motility may range from the slow motion of peritrichous to the darting movement of polar flagellated bacteria.

"Hanging Drop" Method

This method is used to observe the morphology but also demonstrates the motility of organisms. A special slide with a concave center is used or else a ring of plasticine can be placed on the slide. A drop of the culture of bacterial suspension is placed on a coverslip. Vaseline is placed near the concave area of the slide approximately the corners of the coverslip. The slide is placed over the coverslip so that the drop of culture is directly under the concave area and the vaseline adheres to the coverslip.

The slide is then quickly inverted and placed under the microscope. Motile organisms will be seen darting through the medium in which they are suspended.

Fimbria (pili)

Fimbria is term used to designate short, hair-like structures on the surfaces of prokaryotic cells. Like flagella, they are composed of protein (pilin). Fimbriae are shorter and stiffer than flagella, and slightly smaller in diameter. Generally, fimbriae have nothing to do with bacterial movement (there are exceptions, e.g twitching movement on *Pseudomonas*). Fimbriae are very common in Gram-negative bacteria, but occur in some archaea and Gram-positive bacteria as well. Fimbriae are most often involved in adherence of bacteria to surfaces, substrates and other cells or tissues in nature.

In *E. coli*, a special type of fimbria, the **F or sex pili**, mediates the transfer of DNA between mating bacteria during the process of **conjugation**. They are found on "male" bacteria and help in the attachment of those cells to "female" bacteria, forming conjugation tubes through which genetic material is transferred from the donor to the recipient cell.

Common pili (almost always called **fimbriae**) are usually involved in specific adherence (attachment) of procaryotes to surfaces in nature. In medical situations, they are major determinants of bacterial virulence because they allow pathogens to attach to (colonize) tissues and/or to resist attack by phagocytic white blood cells. For example, pathogenic *Neisseria gonorrhoeae* adheres specifically to the human cervical or urethral epithelium by means of its fimbriae; enterotoxigenic strains of *E. coli* adhere to the mucosal epithelium of the intestine by means of specific fimbriae; the M-protein and associated fimbriae of *Streptococcus pyogenes* are involved in adherence and to resistance to engulfment by phagocytes.

Capsules and bacterial protective devices

Most prokaryotes contain some sort of a polysaccharide layer outside of the cell wall polymer. In a general sense, this layer is called a **capsule**. A **true capsule** is a discrete detectable layer of polysaccharides deposited outside the cell wall. A less discrete structure or matrix which embeds the cells is a called a **slime layer** or a **biofilm**. A type of capsule found in bacteria called a **glycocalyx** is a thin layer of tangled polysaccharide fibers which is almost always observed on the surface of cells growing in nature (as opposed to the laboratory). Capsules are often lost during *in vitro* culture.

Detection of bacterial capsules in smear prepared from a pathological material — special staining techniques (**Burry-Hines staining**). **Materials:** 36–48 hour milk culture of *Klebsiella pneumoniae,* India ink*,* crystal violet or basic fuchsine (see figure 13).

Capsules are most pronounced in such bacteria:

- *Klebsiella* (always form the capsules even when growing on simple artificial media).
- *Pneumococci.*
- *Bacilli* causing anthrax.
- *Clostridium perfringens.*
- *Coccobacteria* (excluding Brucella).

Microcapsules (mucopolysaccharide fibrils around the cell wall) are most pronounced in many bacteria and may be detected with electron microscope.

Capsules have **several functions** and often have multiple functions in a particular organism. Like fimbriae, capsules, slime layers, and glycocalyx often **mediate adherence** of cells to surfaces. Capsules also **protect bacterial cells from engulfment** by predatory protozoa or white blood cells (phagocytes), or from attack by antimicrobial agents of plant or animal origin. They also contribute to the virulence of pathogenic bacteria by inhibiting phagocytosis. Loss of the capsule by mutation may render the bacterium avirulent. Capsular material is antigenic and may be demonstrated by serological reactions.

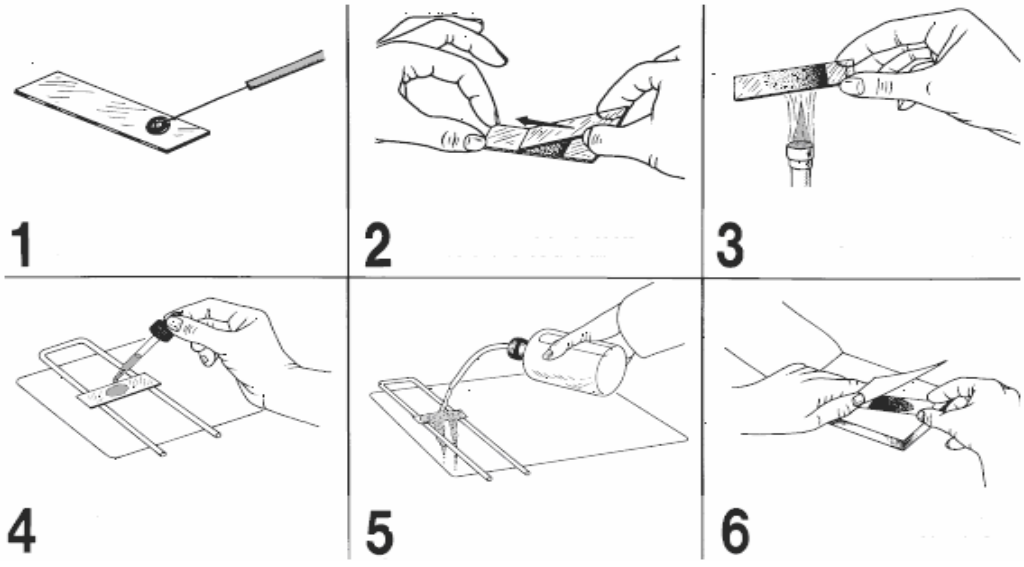


Figure 13 — Procedure for demonstration of capsule presence:
1. Two loopfuls of the organism are mixed in a drop of india inc; 2. The inc suspension of bacteria is spread over slide and air-dried; 3. The slide is gently heat-dried to the organisms to the slide; 4. Smear is stained with crystal violet for one minute; 5. Crystal violet is gently washed off with water; 6. Slide is blotted dry with filtering paper and examined with oil immersion objective.

Capsules in certain soil bacteria **protect cells from effects of drying** or desiccation. Capsular materials may be overproduced when bacteria are fed sugars to become **reserves of carbohydrate** for subsequent metabolism.

Bacteria may attach to surface, produce slime, divide and produce microcolonies within the slime layer, and construct a **biofilm**, which becomes an enriched and protected environment for themselves and other bacteria.

Important characteristic of capsules may be their ability to block some step in the phagocytic process and thereby prevent bacterial cells from being engulfed or destroyed by phagocytes. For example, the primary determinant of virulence of the pathogen *Streptococcus pneumoniae* is its polysaccharide capsule, which prevents ingestion of pneumococci by alveolar macrophages. *Bacillus anthracis* survives phagocytic engulfment because the lysosomal enzymes of the phagocyte cannot initiate an attack on the poly-D-glutamate capsule of the bacterium.

Cell wall

Most procaryotes have a rigid **cell wall**. The cell wall is an essential structure that protects the cell protoplast from mechanical damage and from osmotic rupture or **lysis**.

The cell walls of all **Bacteria** contain a unique type of **peptidoglycan** called **murein**. Peptidoglycan is a polymer of disaccharides cross-linked by short chains of amino acids, and many types of peptidoglycan exist. All **Bacterial** peptidoglycans contain **N-acetylmuramic acid**, which is the definitive component of **murein**.

In the **Gram-positive Bacteria** (those that retain the crystal violet dye when subjected to the Gram-staining procedure), as this is illustrated in figure 14, the cell wall is thick (15–80 nanometers), consisting of several layers of peptidoglycan. In the **Gram-negative Bacteria** (which do not retain the crystal violet) the cell wall is relatively thin (10 nanometers) and is composed of a single layer of peptidoglycan surrounded by a membranous structure called the **outer membrane**. The outer membrane of Gram-negative bacteria invariably contains a unique component, **lipopolysaccharide** (**LPS** or **endotoxin**), which is toxic to animals. In Gram-negative bacteria the outer membrane is usually thought of as part of the cell wall.

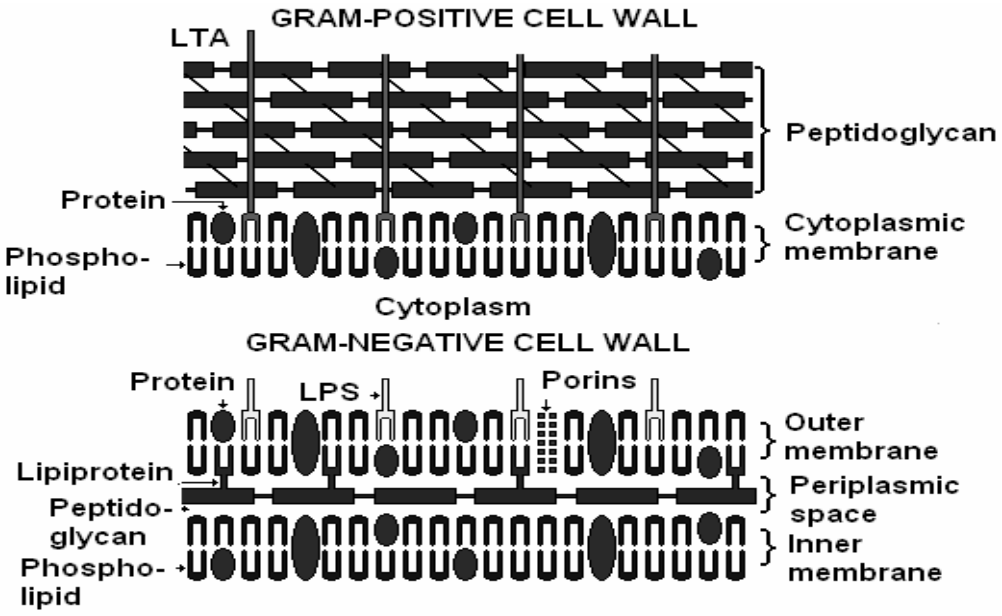


Figure 14 — Profiles of the cell envelope the Gram-positive and Gram-negative bacteria

Peptidoglycan structure and arrangement in *E. coli* is representative of all *Enterobacteriaceae*, and many other Gram-negative bacteria, as well. The glycan backbone is made up of molecules of N-acetylglucosamine and N-acetylmuramic acid molecules, which are cross linked by peptide chains. Then the strands of murein are connected to form a long glycan molecule that encompasses the cell.

The assembly of peptidoglycan (murein) on the outside of the plasma membrane is mediated by a group of periplasmic enzymes which are transglycosylases, transpeptidases and carboxypeptidases. The mechanism of action of penicillin and related beta-lactam antibiotics is to **block transpeptidase and carboxypeptidase enzymes** during their assembly of the murein cell wall. Hence, the beta lactam antibiotic are said to "block cell wall synthesis" in the bacteria.

The glycan backbone of the peptidoglycan molecule can be cleaved by an enzyme called **lysozyme** that is present in animal serum, tissues and secretions, and in the phagocytic lysosome.

The function of lysozyme is to lyse bacterial cells as a constitutive defense against bacterial pathogens. Some Gram-positive bacteria are very sensitive to lysozyme. Gram-negative bacteria are less sensitive to attack by lysozyme because their peptidoglycan is protected by the outer membrane. The exact site of lysozyme is the bond between N-acetylmuramic acid and N-acetylglucosamine.

Closely associated with the layers of peptidoglycan in Gram-positive bacteria are a group of molecules called teichoic acids. **Teichoic acids** are linear polymers of polyglycerol or polyribitol substituted with phosphates and a few amino acids and sugars. The functions of teichoic acid are not known. They are essential to viability of Gram-positive bacteria in the wild.

One idea is that they provide a channel of regularly-oriented negative charges for threading positively charged substances through the complicated peptidoglycan network. Another theory is that teichoic acids are in some way involved in the regulation and assembly of muramic acid subunits on the outside of the plasma membrane. There are instances, particularly in the streptococci, wherein teichoic acids have been implicated in the adherence of the bacteria to tissue surfaces.

Table 2 — Correlation of Grams stain with other properties of bacteria:

Property	Gram-positive	Gram-negative
Thickness of wall	thick (20–80 nm)	thin (10 nm)
Number of peptidoglycan layers	1	2
Peptidoglycan (murein) contents	>50%	10–20%
Teichoic acids in wall	present	absent
Lipid content	0–3%	60%
Protein content	0	9%
Lipopolysaccharide (LPS) content	0	13%
Sensitivity to Penicillin	yes	no (1)
Sensitivity to lysozyme	yes	no (2)

(1) A few Gram-negative bacteria are sensitive to natural penicillins. Many Gram-negative bacteria are sensitive to some type of penicillin, especially semisynthetic penicillins. Gram-negative bacteria, including *E. coli*, can be made sensitive to natural penicillin by procedures that disrupt the permeability characteristics of the outer membrane.

(2) Gram-negative bacteria are sensitive to lysozyme if pretreated by some procedure that removes the outer membrane and exposes the peptidoglycan directly to the enzyme.

The outer membrane of Gram-negative bacteria

Of special interest as a component of the Gram-negative cell wall is the **outer membrane**, a discrete bilayered structure on the outside of the peptidoglycan. For the bacterium, the outer membrane is first permeability barrier and it possesses many interesting and important characteristics of Gram-negative bacteria. The inner face of the outer membrane is composed of phospholipids. The outer face of the outer membrane may contain some phospholipid, but mainly it is formed by a different type of amphiphilic molecule which is composed of lipopolysaccharide (LPS).

The LPS molecule that constitutes the outer face of the outer membrane is composed of a hydrophobic region, called **Lipid A** that is attached to a hydrophilic linear polysaccharide region, consisting of the **core polysaccharide** and **the O-specific polysaccharide** as illustrated in figure 15.

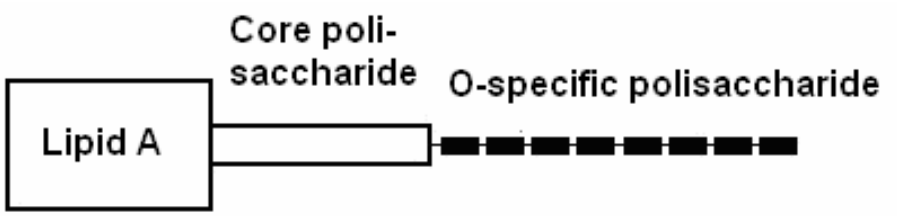


Figure 15 — Structure of LPS

Bacterial lipopolysaccharides are toxic to animals. When injected in small amounts LPS or **endotoxin** activates macrophages to produce pyrogens, activates the complement cascade causing inflammation, and activates blood factors resulting in intravascular coagulation and hemorrhage. Endotoxins may play a role in infection by any Gram-negative bacterium. The toxic component of endotoxin (LPS) is Lipid A. The O-specific polysaccharide may provide ligands for bacterial attachment and confer some resistance to phagocytosis. Variation in the exact sugar content of the O polysaccharide (also referred to as the O antigen) accounts for multiple antigenic types (serovariants) among Gram-negative bacterial pathogens.

The cell walls of bacteria deserve special attention for several reasons:

1. They are an essential structure for viability.
2. They are composed of unique components found nowhere else in nature.
3. They are one of the most important sites for attack by antibiotics.
4. They provide ligands for adherence and receptor sites for drugs or viruses.
5. They cause symptoms of disease in animals.
6. They provide for immunological distinction and variation among strains of bacteria.

Gram staining

In 1884 the Danish bacteriologist Christian Gram developed a staining technique that separates bacteria into two groups: those that are gram-positive and those that are gram-negative. The procedure is based on the ability of microorganisms to retain the purple color of crystal violet during decolourization with alcohol. Gram-negative bacteria are decolorized by the alcohol, losing the purple color of crystal violet. Gram-positive bacteria are not decolorized and remain purple. After decolourization, safranin (fuchsine), a red counterstain, is used to impart a pink color to the decolorized gram-negative organisms.

Figure 17 illustrates the effects of the various reagents on bacterial cells at each stage in the process. Note that crystal violet, the **primary stain,** causes both gram-positive and gram-negative organisms to become violet after 2 minutesof staining.

When Gram's iodine, the **mordant,** is applied to the cells for one minute, the color of gram-positive and gram-negative bacteria remains the same: purple. The function of the mordant here is to combine with crystal violet to form a relatively insoluble compound in the gram-positive bacteria. When the **decolorizing agent,** 95% ethanol, is added to the cells for 10–20 seconds, the gram-negative bacteria become colorless, but the gram-positive bacteria remain purple.

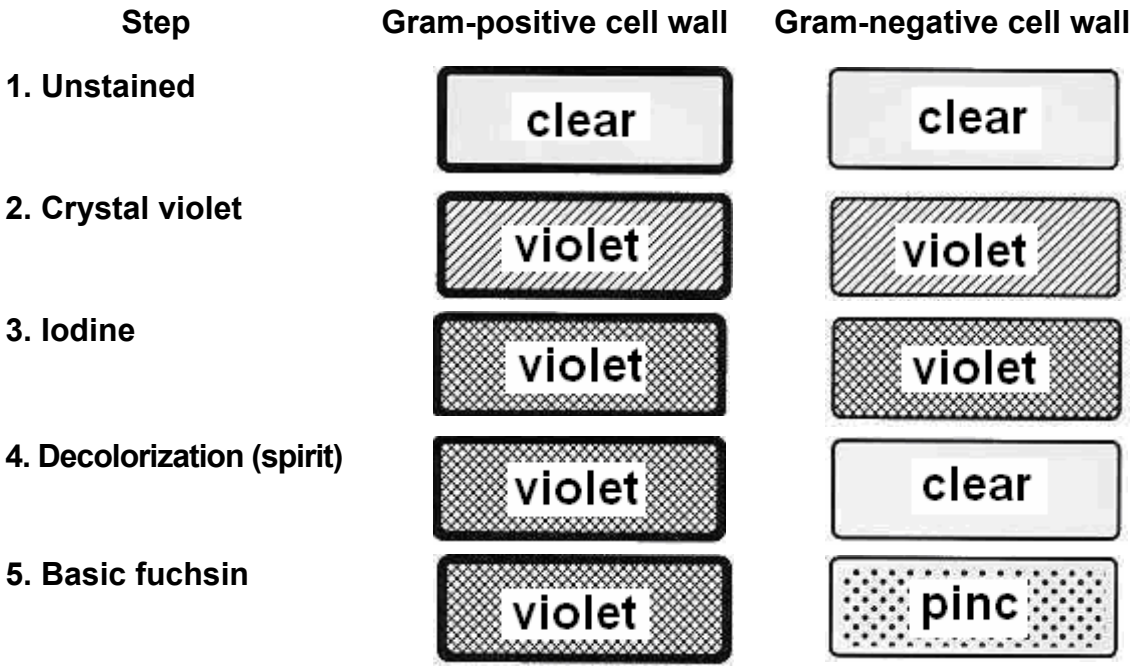


Figure 17 — Procedure of Gram staining

In the final step a **counterstain,** safranin (fuchsine), adds a pink color to the decolorized gram-negative bacteria without affecting the color of the purple gram-positive bacteria.

STAINING PROCEDURE consists of 8 steps:

1. Cover the smear with **crystal violet** and let stand for *2 minutes.*
2. Briefly wash off the stain, using a wash bottle of distilled water.
3. Cover the smear with **iodine** solution and let it stand for *one minute.*
4. Pour off the Gram's iodine and flood the smear with **95% ethyl alcohol** for *10 to 20 seconds.* This step is critical. Thick smears will require more time than thin ones. *Decolourization has occurred when the solvent flows colorlessly from the slide.*
5. Stop action of the alcohol by rinsing the slide with water from wash bottle for a *few seconds.*
6. Cover the smear with **basic fuchsine** for *2–3 minutes.*
7. Wash gently for a few seconds, blot dry with bibulous paper, and air-dry.
8. Examine the slide under oil immersion.

The exact mechanism of the Gram reaction is not understood. The Gram-positive cells have more acidic protoplasm, which may account for their retaining the primary stain more strongly than the Gram-negative bacteria. The Gram-positive bacteria become

Gram-negative when the cell wall is damaged. Gram-positive and Gram-negative bacteria differ not only in staining characteristics and in structure but also in several other properties such as growth requirements, sensitivity to antibiotics and pathogenicity!

Table 3 — Differentiation of the bacteria according to their gram-state

Gram-negative group of bacteria	Gram-positive group of bacteria
1. Exclusion from cocci: diplococci — *Neisseria Gonococcus and Neisseria Meningococcus*	**1**. **Majority of cocci:** *Streptococci, Staphylococci, Sarcina, Pneumococci*
2. Non spore-forming rods (true bacteria): *Enterobacteria (exp. E. coli, S. Typhi)*	**2. Spore-forming rods:** *Bacilli and Clostridia*
3. Spiral forms of bacteria: *Spirochetes (Leptospira, Borrelia, Treponema), Spirilla, Vibrio*	**3. Rods with irregular form:** *Corynebacteria and Mycobacteria*
4. Atypical forms of bacteria: *Rickettsia and Chlamydia*	**4. Rods with regular form:** *Listeria*
5. Bacteria lost cell wall and bacteria with defect of cell wall: *Mycoplasma, L-forms*	**5. Branch-forming rods:** *Actinomycetes: center of bacteria (druse) is Gram-positive, but peripheral parts (thickened fibers) is Gram-negative*

Plasma membrane

The **plasma membrane**, also called the **cytoplasmic membrane**, is the most dynamic structure of a prokaryotic cell. Its main function is **selective permeability barrier** that regulates the passage of substances into and out of the cell by means special membrane **transport processes** and **transport systems**.

Functions of the prokaryotic plasma membrane:

1. Osmotic or permeability barrier.
2. Location of transport systems for specific solutes.
3. Energy generating functions, involving respiratory and photosynthetic electron transport systems.
4. Synthesis of membrane lipids (including LPS in Gram-negative cells).
5. Synthesis of cell wall peptidoglycan.
6. Coordination of DNA replication and segregation with septum formation and cell division.
8. Chemotaxis.
9. Location of specialized enzyme system.

Transport processes

There are four general transport mechanisms involved in membrane transport:

1. **Facilitated diffusion**: passive diffusion of a substrate against a concentration gradient and does not require energy (glycerol);
2. **Binding protein dependent transport**: with help of specific binding proteins located in a periplasmic space in Gram-negative bacteria and requires ATP;
3. **Chemiosmotic-driven transport**: movement a molecule across the plasma membrane at the expense of ion gradient such as proton-motive or sodium-motive force. The proteins that mediate the passage of solutes through membrane are called **carrier proteins** or **permeases**. Transport systems operate by one of three **trans-**

port processes as described below in Figure. In a **uniport** process, a solute passes through the membrane unidirectionally. In **symport** processes two solutes must be transported in the same direction at the same time; in **antiport** processes one solute is transported in one direction simultaneously as a second solute is transported in the opposite direction (see figure 18).

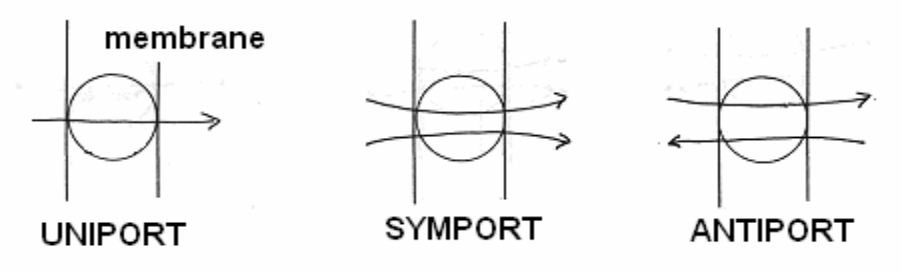


Figure 18 — Transport processes in bacterial cells

4. **Group translocation**: this process allows bacteria to utilize their energy resources efficiently by coupling transport with metabolism (substrate such as free sugar becoming phosphorilated during the transport system).

The plasma membrane of procaryotes may invaginate into the cytoplasm or form vesicles attached to the inner membrane surface. These structures are sometimes referred to as **mesosomes.** Such internal membrane systems may be analogous to the mitochondria or the chloroplasts which increase the surface area of membranes to which enzymes are bound for specific enzymatic functions. The photosynthetic apparatus (light harvesting pigments and ATPase) of photosynthetic procaryotes is contained in these types of membranous structures. Mesosomes may also represent specialized membrane regions involved in DNA replication and segregation, cell wall synthesis, or increased enzymatic activity. There are a few antibiotics (e.g. polymyxin), hydrophobic agents (e.g. bile salts), and proteins (e.g. complement) that can damage bacterial membranes.

Cell wall-less forms and L-transformation

A few bacteria are able to live or exist without a cell wall. The **mycoplasmas** are a group of bacteria that lack a cell wall. Mycoplasmas have sterol-like molecules incorporated into their membranes and they are usually inhabitants of osmotically-protected environments. *Mycoplasma pneumoniae* is the cause of primary atypical bacterial pneumonia, known in the vernacular as "walking pneumonia". For obvious reasons, penicillin is ineffective in treatment of this type of pneumonia.

Cell wall synthesis may be inhibited by many factors. Lysozyme (enzyme normally present in many tissue fluids) lyses sensitive bacteria by splitting the cell wall mucopeptide links. When lysozyme acts on a Gram-negative bacterium in a hypertonic solution, a **protoplast** is formed (bacterium without cell wall). With Gram-positive bacteria, the result is a **spheroplast** which differs from the protoplast in that some cell wall material is retained. Protoplast and spheroplast are spherical, regardless of the original shape of the bacterium. Cell wall deficient forms of bacteria may have a role in the persistence of certain chronic infections such as pyelonephritis.

If cell wall-less cells are able to grow and divide, they are called **L-forms**, after Lister Institute, London, where the observation was made. L-forms are seen in several species of bacteria. L-forms are difficult for cultivation and usually require a medium that is solidified with agar as well as having the right osmotic strength. L-form

may be **unstable** in that this morphological defect is maintained only in presence of penicillin or other agents that interfere with the cell wall synthesis. Thus, they are able to resume normal cell wall synthesis. Also L-forms may be stable, when this defect becomes the constant feature of the strain and is retained in serial generations.

Some bacteria species produce L-forms spontaneously. The spontaneous or antibiotic-induced formation of L-forms in the host may produce chronic infections (persistent infections). Since L-forms infections are relatively resistant to the antibiotic treatment, they present special problems in chemotherapy. Their reversion to the bacillary form can produce relapses of the infection.

Universal L-transformating agents:

1. Antibiotics
2. Aminoacids
3. Lysozyme
4. Physical factors

Main properties of L-forms:

1. Ability to persist in the body during a long time;
2. Presence stable and unstable forms, which are able to reverse in each other (reversion);
3. Low degree of virulence;
4. Change of antigenic structure (absence O- and K-antigens);
5. Loss of mesosomes after reversion;
6. Was reduced a n amount of nucleic acids;
7. Was increased an amount of lipids.

Cytoplasm

The cytoplasmic constituents of prokaryotic cells invariably include the **prokaryotic chromosome (nucleoid)** and **ribosomes**. The chromosome is typically one large circular molecule of **DNA**, more or less free in the cytoplasm. This DNA is not associated with basic protein and has no nuclear membrane. The total DNA content of a Procaryote is referred to as the cell **genome**. The bacterial chromosome is haploid and replicates by simple (binary) fission instead of by mitosis as in eukaryotes. During cell growth and division, the prokaryotic chromosome is replicated in the usual **semi-conservative fashion** before for distribution to progeny cells.

Procaryotes sometimes possess smaller extrachromosomal pieces of DNA called **plasmids**. They are not essential for the life of the cell they inhabit but may confer on it certain properties like Toxigenicity and drug resistance which may constitute a survival advantage.

Genotype is set of all genes in the cell. **Plasmotype** is set of extrachromosomal genes. **True genotype** is set of chromosomal genes.

The distinct granular appearance of prokaryotic cytoplasm is due to the presence and distribution of **ribosomes.** The ribosomes of procaryotes are smaller than cytoplasmic ribosomes of eukaryotes. Prokaryotic ribosomes are 70S in size, being composed of 30S and 50S subunits. Ribosomes are involved in the process of translation (protein synthesis). Bacterial chromosome, plasmids and ribosomes may be seen by electron microscopy.

Inclusions are distinct granules that may occupy a substantial part of the cytoplasm. Inclusion granules are usually reserve materials of some sort. For example, carbon and energy reserves may be stored as glycogen (a polymer of glucose) or as polybetahydroxybutyric acid (a type of fat) granules. Elemental sulfur (sulfur globules) are stored by some phototrophic and some lithotrophic procaryotes as reserves of energy or electrons. They are characteristic for different species and depend on the age and condition of the culture.

Volutine granules are highly refractive, strongly basophilic bodies consisting of polymetaphophate. Special **staining techniques** such as **Neisser's** demonstrate granules very clearly. These granules are characteristically present in diphtheria bacilli. They have been considered to represent a reserve of energy and phosphate for cell metabolism but they are most frequent in cells grown under conditions of nutritional deficiency and tend to disappear when the deficient nutrients are supplied.

Endospores and endospore formation

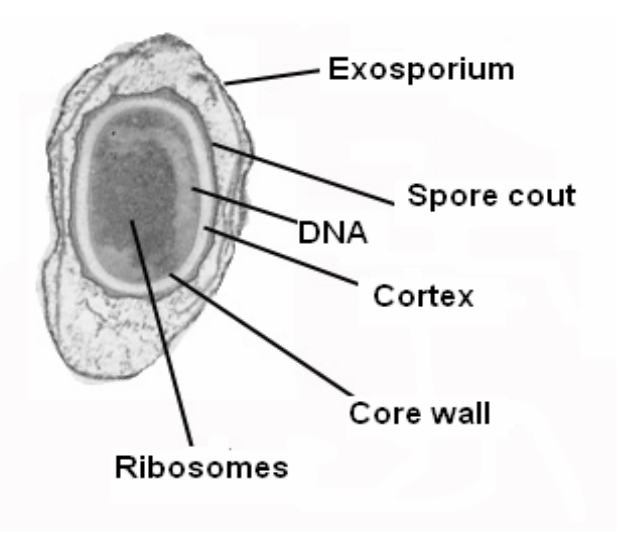


Figure 19 — Structure of spore

Spores are highly resistant to environmental stresses such as high temperature (some endospores can be boiled for hours and retain their viability; spores of all medically important species are destroyed by autoclaving at 120ºC for 15 minutes!), irradiation, strong acids, disinfectants, etc. Spores are probably the most durable (resting) cells produced in nature.

Endospores are formed by vegetative cells in response to environmental signals that indicate a limiting factor for vegetative growth, such as exhaustion of an essential nutrient.

They germinate and become vegetative cells when the environmental stress is relieved. Hence, endospore-formation is a mechanism of survival rather than a mechanism of reproduction. Sheme of spore is illustrated in figure 19.

Function: defense from unfavorable physical-chemical factors of external environment and attrition of a medium. **Composition:** DNA, covered with multilayer spore coat containing peptidoglycan (cortex). The spore has a core wall of unique peptidoglycan surrounded by several layers, including the cortex, the spore coat and the exosporium. The dehydrated core contains the bacterial chromosome and a few ribosomes and enzymes to protein synthesis and metabolism during germination.

Table 4 — Differences between endospores and vegetative cells:

Property	Vegetative cells	Endospores
Surface coats	Typical Gram-positive murein cell wall polymer	Thick spore coat, cortex, and peptidoglycan core wall
Microscopic appearance	Nonrefractile	Refractile
Enzymatic activity	Present	Absent
Macromolecular synthesis	Present	Absent
Heat resistance	Low	High
Resistance to chemicals and acids	Low	High
Radiation resistance	Low	High
Sensitivity to lysozyme	Sensitive	Resistant
Sensitivity to dyesand staining	Sensitive	Resistant

Spore-forming bacteria: member of genera *Bacillus* (spore doesn't exceed a cell diameter) and *Clostridium* (size of the spore is bigger then a cell diameter). The shape and position of the spore and its size relative to parent cell are **species characteristics**! Spore may be central, terminal or subterminal (see figure 20). They may be oval or spherical.

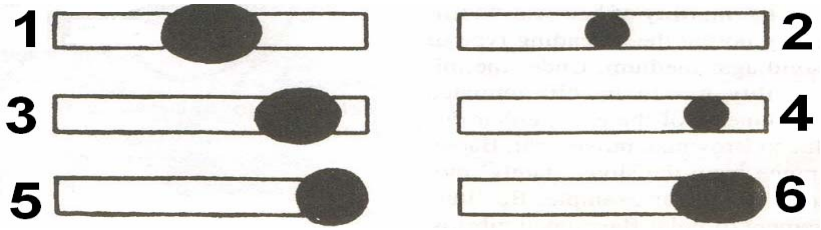


Figure 20 — Types of bacterial spores:
1. Central, bulging; **2**. Central, not bulging; **3.** Subterminal, bulging;
4. Subterminal, not bulging; **5**. Terminal, spherical; **6.** Terminal, oval

During **endospore formation** (see figure 21), a vegetative cell is converted to a heat-resistant spore. There are eight stages in the sporulation cycle of a *Bacillus* species, and the process takes about eight hours. During the early stages one bacterial chromosome and a few ribosomes are partitioned off by the bacterial membrane to form a protoplast within the mother cell. By the late stages the protoplast has developed a second membrane and several wall-like layers of material are deposited between the two membranes. **Conditions for sporulation:** external environment (not in human organism) and artificial nutritive media.

Exospores are reproductive structures in streptomycetes.

Differences in compare with endospores:

1. Not resistant in unfavorable conditions of external environment
2. Forms outside of the bacterial cell
3. One bacterial cell contains many (not single) exospores.

The factors ensuring thermal resistance:

1. Practical absence of unbound water;
2. Increased calcium concentration;
3. Presence of dipicolinic acid;
4. Especial composition of protein;
5. Especial composition of peptidoglycan of the cortex.

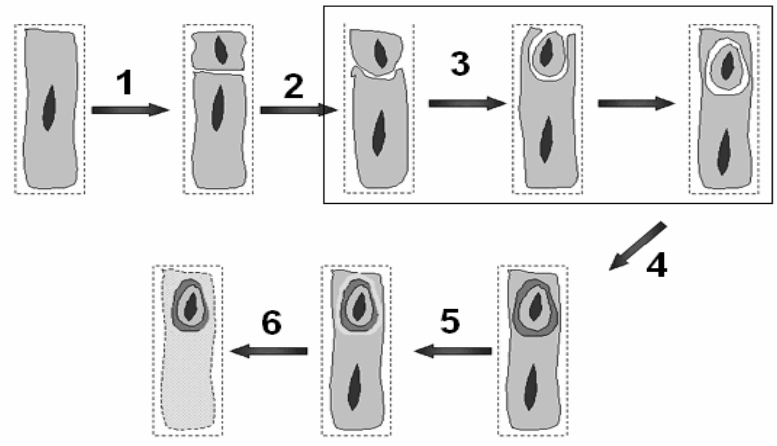


Figure 21 — Scheme of endospore formation:
1. End of vegetative growth; **2.** Asymmetric septation; **3**. Endocytosis or enulfment of daughter protoplast; **4.** Lysis of mother cell and release of SPORE; **5.** Assemble of spore coat;
6. Synthesis of peptidoglycan-containing cortex.

Detection of spores in bacterial cells

Species of bacteria, belonging principally to the genera *Bacillus* and *Clostridium,* produce extremely heat-resistant endospores. In addition to being heat-resistant, they are very resistant to many chemicals that destroy non-spore-forming bacteria. This resistance to heat and chemicals is due primarily to a thick, tough spore coat.

Staining of Spores (Modified Ziehl Nielsen's method):

1. Stain with *carbolfuchsin* for 5–10 minutes, heating until steam rises.
2. Wash in tap water.
3. Decolourize with 0.5% *sulfuric acid* or methylated spirit. If the acid is stronger than 1%, spores of many bacilli are decolourized.
4. Wash in tap water. Now the smear is examined and if both bacilli and spores are red, it is decolourized again. If the spores alone are stained, it is counterstained. Let the *counterstain* to act for 2 minutes. Wash in water, blot and dry.

Result: the spores are stained bright red and the bacilli blue.

Ziehl-Nielsen staining for acid-fast bacteria: This stain is another method of categorizing certain bacteria, depending on their ability to resist decolourization by acid and alcohol. A very strong stain is used, basic fuchsine in a phenol solution and heat is applied in order that the stain can penetrate the waxy covering certain bacteria. Most bacteria in the genus *Mycobacterium* contain considerable amounts of wax like lipoid material, which affects their staining properties. Unlike most other bacteria, once they are properly stained with carbolic fuchsine, they resist decolourization with acid alcohol. Since they are not easily decolorized they are said to be acid-fast. This property sets them apart from many other bacteria.

Method for acid-fast bacteria:

1. Make a smear of the material and allow drying at room temperature.
2. Flood the whole slide with strong carbolfuchsin and heat the slide until steam is seen rising from the slide.
3. Rinse in water and flood the slide with 25% sulfuric acid. Leave this until the smear is pale pink in color.
4. Rinse in water and pour on alcohol for a few minutes.
5. Counterstain with methylene blue.
6. Dry by standing the slide.

Results: the tubercle bacillus resists decolorizing by acid and alcohol (i.e., it is both acid and alcohol fast) it will remain bright red while all other organisms and material will take on the color of the counterstain.

MORPHOLOGY AND STRUCTURE OF SPIROCHETES

Taxonomy of Spirochetes:

KINGDOM: *Procaryote*
DIVISION: *Gracilicutes*
ORDER: *Spirochaetales*
FAMILY: **Spirochaetaceae** GENUS:
Treponema,
Borrelia
Leptospiraceae GENUS:
Leptospira
SPECIES: ***Treponema pallidum*** (syphilis causal organism)
Borrelia recurrentis (relapsing fever causal organism)
Leptospira interrogans (canicola fever /leptospirosis/ causal organism)

Treponema: Some species are pathogenic and parasitic for humans and other animals, generally producing local lesions in tissues. 8–12 coils having regular amplitude. *T. pallidum* is the causative agent of **syphilis**, a common sexually-transmitted disease found world-wide. Multiplication is by binary fission. Treponemes have not yet been cultured in vitro. Traditionally this organism has been considered a strict anaerobe, but it is now known to be microaerophilic. The composition of *T.pallidum* is approximately 70 percent proteins, 20 percent lipids, and 5 percent carbohydrates. This lipid content is relatively high for bacteria (see figure 22).

The lipid composition immunologically distinct from lipopolysaccharide. Although treponemes possess both outer and cytoplasmic membranes they differ considerably in structure from enteric Gram-negative bacteria.

The organism has an outer membrane containing an extremely low density of transmembrane proteins. Typically, three flagella originate from each end of the bacterium. Unlike Gram-negative bacteria in which the peptidoglycan underlies the outer membrane, in treponemes the murein layer overlies the cytoplasmic membrane. The cytoplasmic membrane covers the protoplasmic cylinder; this membrane contains the majority of the bacterium's integral membrane proteins and is particularly lipoproteins.

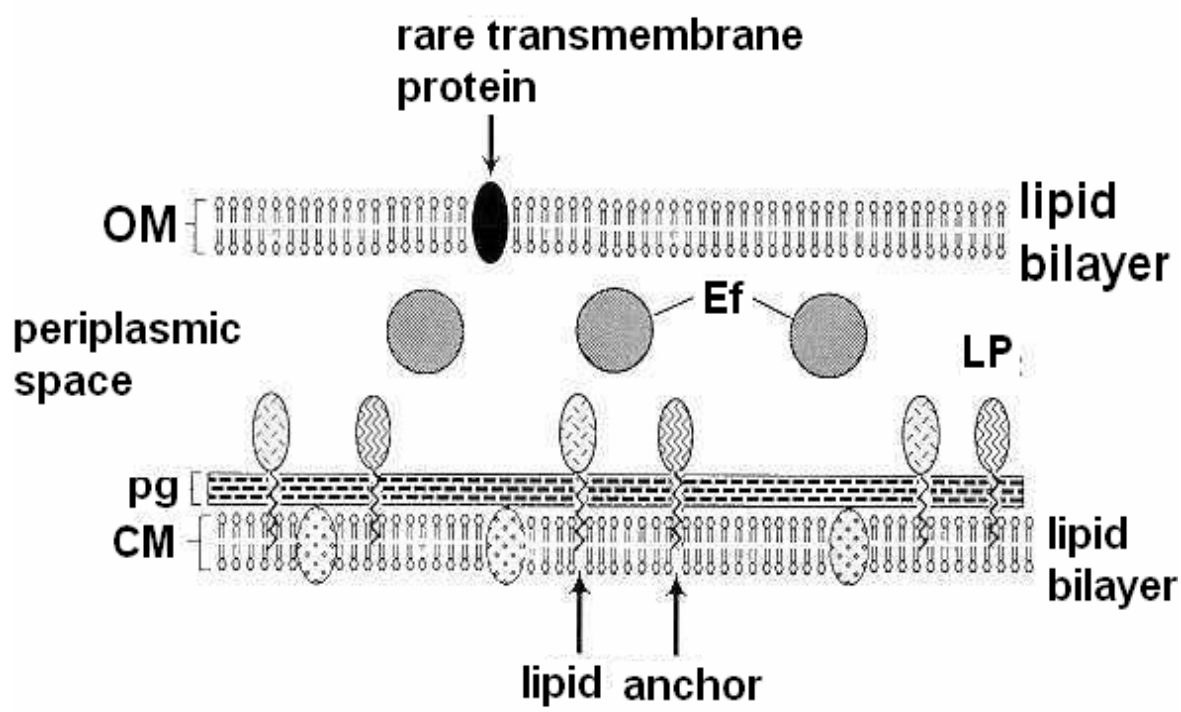


Figure 22 — Proposed structure of treponemal outer and cytoplasmic membranes (abbreviations:
OM — outer membrane; Ef — endoflagella or periplasmic flagella;
LP — lipoproteins; pg — peptidoglycan; CM — cytoplasmic membrane).

Leptospira is a flexible, spiral-shaped, Gram-negative spirochete with internal flagella. *Leptospira interrogans* causes leptospirosis, a usually mild febrile illness that may result in liver or kidney failure. Aerobic bacteria**.** The cell is encased in a three- to five-layer outer membrane or envelope. Outer membranes are the flexible, helical peptidoglycan layer and the cytoplasmic membrane; these encompass the cytoplasmic contents of the cell. The structures surrounded by the outer membrane are collectively called the **protoplasmic cylinder.**

The thin (0. 1 μm by 8 to 20 μm) leptospires are coiled, flexible cells. In liquid media, one or both ends are usually hooked. Leptospires are too thin to be visualized with the light microscope but are clearly seen by dark-field or phase contrast microscopy. The leptospires have two periplasmic flagella, one originating at each end of the cell. *Leptospira* differs from other spirochetes in lacking glycolipids (see figure 23).

Borrelia: These organisms are parasitic on many forms of animal life, are generally hematophytic, or are found on mucous membranes. Some borreliae are transmitted by the bites of arthropods. Like *Leptospira*, *Borrelia* is a flexible, spiral-shaped, Gram-negative spirochete with internal flagella. *Borrelia recurrentis* (louse borne) cause relapsing fevers: influenza-like febrile diseases that follow a relapsing and remitting course. *Borrelia* has morphologic characteristics similar to those of *Leptospira,* except that cells average 0.2 to 0.5 μm by 4 to 18 μm and have fewer coils (see figure 23).

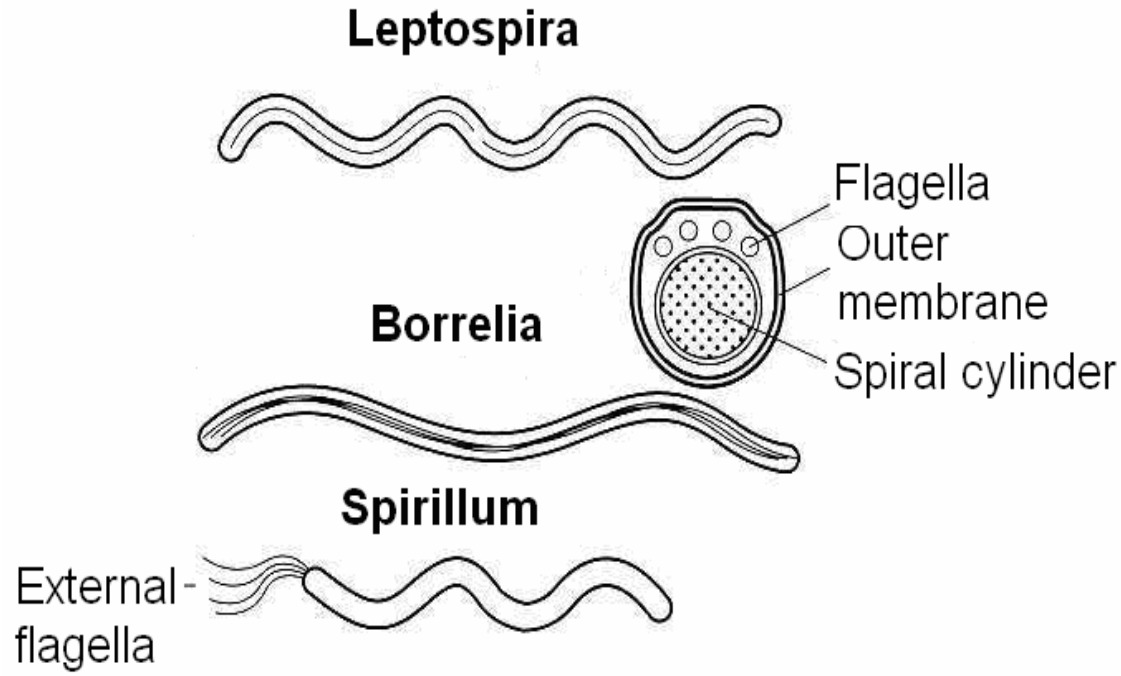


Figure 23 — Structure of spirochetes

Seven to twenty periplasmic flagella originate at each end and overlap at the center of the cell. Because of their larger diameter, borreliae are more readily stained with aniline dyes than are other spirochetes. Their lipid components are unusual in that they include cholesterol; this substance has been found in only one other bacterial genus, *Mycoplasma.*

Spirochetes are procaryotes similar with Protists. Obligate intracellular parasites. Structurally, a spirochete consists of a rod of protoplasm bound by a cell wall and outer membrane. The organ of motility is an axial filament (fibril) which is localized in periplasmic space of the cell wall and situated along the cell (see figure 24). The filament is built of contractive protein flagellin (the same as flagella). Therefore spirochetes move by the way of contraction of the body of bacterial cell. An unusual feature of the spirochetes is the location of the flagella, which lie between the outer membrane and the peptidoglycan layer. They are referred to as periplasmic flagella. The periplasmic flagella are attached to the protoplasmic cylinder subterminally at each end and extend toward the center of the cell. The number of periplasmic flagella per cell varies among the spirochetes. The motility of bacteria with external flagella is impeded in viscous environments, but that of spirochetes is enhanced.

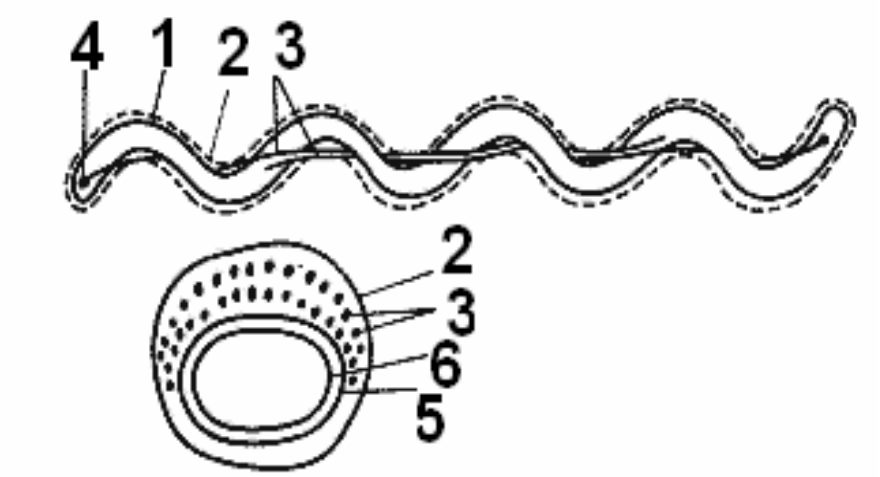


Figure 24 — 1. Protoplasmic cylinder; 2. Outside cover; 3. Axial fibrils; 4. Place of localization of axial fibrils; 5. Peptidoglycan layer of the cell wall; 6. Cell membrane

Method of detecting of Spirochetes:

1. In native (uncolored) preparations – study of mobility with using dark field microscopy ("hanging drop");
2. Romanovsky-Giemsa staining (Treponema – pink; Leptospira – red; Borrelia – blue);
3. Gram-staining (Spirochetes give gram-negative reaction).

MORPHOLOGY AND STRUCTURE OF MYCOPLASMA

Taxonomy of Mycoplasma:

KINGDOM: *Procaryote*

DIVISION: *Tenericutes*

CLASS: *Mollicutes*

ORDER: *Mycoplasmatales*

FAMILY: *Mycoplasmataceae*

GENUS *Mycoplasma and* Ureaplasma

SPECIES: ***Mycoplasma pneumoniae*** — agent of pneumonia

Ureaplasma urealyticum — agent of non-gonococcal urethritis

Study techniques:

1. Phase contrast microscopy
2. Electron microscopy.

The mycoplasmas are the smallest free-living bacteria. They range from 0.2–0.8 micrometers and thus can pass through some filters used to remove bacteria. They have the smallest genome size and, as a result, lack many metabolic pathways and require complex media for their isolation. The mycoplasmas are **facultative anaerobes**, except for *M. pneumoniae*, which is a **strict aerobe**. A characteristic feature that distinguishes the mycoplasmas from other bacteria is the lack of a cell wall. Thus, they can assume multiple shapes including round, pear shaped and even filamentous. Mycoplasmas are incapable of synthesizing sterols for cytoplasmic membrane — **"membrane parasites"**.

MORPHOLOGY AND STRUCTURE OF RICKERRSIA AND CHLAMYDIA

Taxonomy of Rickettsia and Chlamydia:

KINGDOM: *Procaryote*

DIVISION: *Gracilicutes*

ORDER: *Rickettsiales*

FAMILY: *Rickettsiaceae*

GENUS *Rickettsia; Bartonella; Coxiella*

SPECIES: ***Rickettsia prowazekii*** — agent of epidemic

Coxiella burnetii — agent of Q-fever

KINGDOM: *Procaryote*

DIVISION: *Gracilicutes*

ORDER: *Chlamydiales*

FAMILY: *Chlamydiaceae*

GENUS *Chlamydia*

SPECIES: ***Chlamydia trachomatis*** – agent of urogenital infections, trachoma, conjunctivitis, pneumonia and lymphogranuloma venereum

Chlamydophila pneumoniae – agent of bronchitis, sinusitis, pneumonia and possibly atherosclerosis

The **Rickettsiae** are small, Gram-negative, aerobic, coccobacilli that are **obligate intracellular parasites** of eukaryotic cells. They may reside in the cytoplasm or within the nucleus of the cell that they invade. They divide by binary fission and they metabolize host-derived glutamate via aerobic respiration. They have typical Gram-negative cell walls and they lack flagella. The Rickettsiae frequently have a close relationship with arthropod vectors that may transmit the organism to mammalian hosts. Rickettsia must be grown in the laboratory by co-cultivation with eukaryotic cells, and they have not been grown by in artificial medium.

Chlamydiae are small **obligate intracellular parasites** and were once considered to be viruses. However, they contain DNA, RNA and ribosomes and make their own proteins and nucleic acids and are now considered to be true bacteria. They possess an inner and outer membrane similar gram-negative bacteria and a lipopolysaccharide but do not have a peptidoglycan layer. Although they synthesize most of their metabolic intermediates, they are unable to make their own ATP and thus are **energy parasites**. Chlamydia — cocci, which are presented as cytoplasm inclusion bodies (microcolonies, covered by coat having origin from the host cell membrane).

The developmental cycle of Chlamydia

Studies on the growth cycle of *C trachomatis* in cell cultures in vitro revealed that the infectious elementary body develops into a noninfectious reticulate body (RB) within a cytoplasmic vacuole in the infected cell. There is an eclipse phase of about 20 hours after entry of the elementary body into the infected cell, during which the infectious particle develops into a reticulate body. In these structures the chlamydial ge-

nome is transcribed into RNA, proteins are synthesized, and the DNA is replicated. The reticulate body divides by binary fission to form particles which, after synthesis of the outer cell wall, develop into new infectious elementary body progeny. The yield of chlamydial elementary bodies is maximal 36 to 50 hours after infection (see figure 25).

Method of detection of Rickettsia and Chlamydia:

1. Romanovsky-Giemsa staining (dark blue bacteria on the light blue background of the cell).
2. Zdradovsky staining (pink bacteria on the light blue background of the cell).
3. Gram-staining (gram-negative reaction).

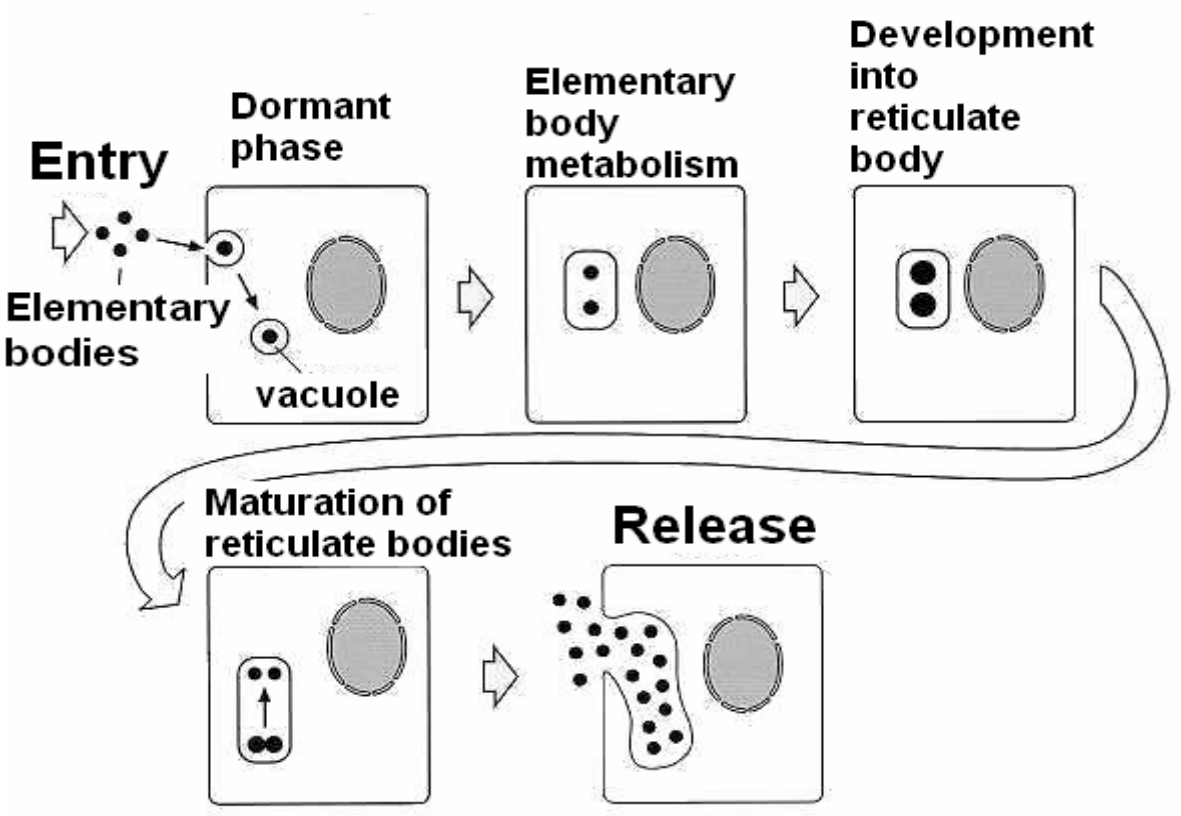


Figure 25 — Life cycle of Chlamydia

MORPHOLOGY AND STRUCTURE OF ACTINOMYCETES

In this section, we shall discuss three genera of actinomycetes: **Actinomyces, Nocardia**, and **Streptomyces**. These organisms have been shown to be higher bacteria, but they were thought to be fungi for many years because they have filamentous forms, 0.5 to 0.8 microns in diameter, which appear to branch. Some species form aerial mycelia in culture. The clinical manifestations of infection are similar to those of a systemic fungal infection. It is now clear that they are not fungi but are closely related to the mycobacteria. Actinomyces are anaerobic, while Nocardia and Streptomyces are aerobic. Nocardia stain partially acid-fast, Actinomyces and Streptomyces are not acid-fast. Actinomyces produce granules (**druses**). Peptidoglycan of the cell wall contains sugars which are not present in other Prokaryotes.

The most common cause of **actinomycosis** is the organism *Actinomyces israelii* which infects both man and animals. In cattle, the disease is called "lumpy jaw" because of the huge abscess formed in the angle of the jaw. In man, *A. israelii* is an

endogenous organism that can be isolated from the mouths of healthy people. Pus will be the clinical material for the laboratory. If you rotate the vial of pus, the yellow sulfur granules can be seen with the naked eye (druses). The organism is a **Gram positive rod** (only center of druse!) that frequently branches.

The most common species of **Nocardia** which cause disease in human beings are *N. brasiliensis* and *N. asteroides*. These are soil organisms which can also be found endogenously in the sputum of apparently healthy people. *N. asteroides* is usually the etiologic agent of pulmonary nocardiosis. These organisms rarely form granules. The Nocardia are aerobic, gram-positive rods and stain partially acid-fast (i.e., the acid-fast staining is not uniform).

The **streptomyces** species usually cause the disease known as mycetoma (fungus tumor). The streptomycetes are aerobic like Nocardia, and can grow on both bacterial and fungal media. They produce a chalky aerial mycelium with much branching. The various species of streptomyces produce granules of different size, texture and color. These granules along with colonial growth and biochemical tests allow the bacteriologist or mycologist to identify each species. Produce antibiotics.

Mycology

Fungi are eukaryotic organisms that do not contain chlorophyll, but have cell walls, filamentous structures, and produce spores. These organisms grow as saprophytes and decompose dead organic matter. There are between 100,000 to 200,000 species depending on how they are classified. About 300 species are presently known to be pathogenic for man.

The taxonomy of the Kingdom Fungi is evolving and is controversial. Formerly based on gross and light microscopic morphology, studies of ultra structure, biochemistry and molecular biology provide new evidence on which to base taxonomic positions. Medically important fungi are in four phyla:

1. **Ascomycota**: Sexual reproduction in a sack called an ascus with the production of ascopspores.
2. **Basidiomycota**: Sexual reproduction in a sack called a basidium with the production of basidiospores.
3. **Zygomycota**: Sexual reproduction by gametes and asexual reproduction with the formation of zygospores.
4. **Mitosporic Fungi** (Fungi Imperfecti): No recognizable form of sexual reproduction. Includes most pathogenic fungi.

Pathogenic fungi can exist as yeasts or as hyphae. A mass of hyphae is called **mycelia**. Yeasts are unicellular organisms and mycelia are multicellular filamentous structures, constituted by tubular cells with cell walls. The yeasts reproduce by budding. The mycelial forms branch and the pattern of branching is an aid to the morphological identification. If the mycelia do not have SEPTA, they are called coenocytic (nonseptate). The terms "hypha" and "mycelium" are frequently used interchangeably. Some fungi occur in both the yeast and mycelial forms. These are called dimorphic fungi.

The dimorphic fungi have two forms:

1. YEAST (parasitic or pathogenic form): This is the form usually seen in tissue, in exudates, or if cultured in an incubator at 37 degrees C.

2. MYCELIUM (saprophytic form): The form observed in nature or when cultured at 25 degrees C. Conversion to the yeast form appears to be essential for pathogenicity.

Fungi are identified by several morphological or biochemical characteristics, including the appearance of their fruiting bodies. The asexual spores may be large (macroconidia, chlamydospores) or small (microconidia, blastospores, arthroconidia).

There are four types of mycotic diseases:

1. **Hypersensitivity** — an allergic reaction to molds and spores.
2. **Mycotoxicoses** — poisoning of man and animals by feeds and food products contaminated by fungi which produce toxins from the grain substrate.
3. **Mycetismus** — the ingestion of toxin (mushroom poisoning).
4. **Infection.**

We shall be concerned only with the last type: pathogenic fungi that cause infections. Most common pathogenic fungi do not produce toxins but they do show physiologic modifications during a parasitic infection (e.g., increased metabolic rate, modified metabolic pathways and modified cell wall structure). The mechanisms that cause these modifications as well as their significance as a pathogenic mechanism are just being described. Most pathogenic fungi are also thermotolerant, and can resist the effects of the active oxygen radicals released during the respiratory burst of phagocytes. Thus, fungi are able to withstand many host defenses. Fungi are ubiquitous in nature and most people are exposed to them. The establishment of a mycotic infection usually depends on the size of the inoculum and on the resistance of the host. The severity of the infection seems to depend mostly on the immunologic status of the host. Thus, the demonstration of fungi, for example, in blood drawn from an intravenous catheter can correspond to colonization of the catheter, to transient fungemia (i.e., dissemination of fungi through the blood stream), or to a true infection. The physician must decide which is the clinical status of the patient based on clinical parameters, general status of the patient, laboratory results, etc. The decision is not trivial, since treatment of systemic fungal infections requires the aggressive use of drugs with considerable toxicity. Most mycotic agents are soil saprophytes and mycotic diseases are generally not communicable from person-to-person (occasional exceptions: Candida and some dermatophytes). Outbreaks of disease may occur, but these are due to a common environmental exposure, not communicability. Most of the fungi which cause systemic infections have a peculiar, characteristic ecologic niche in nature. This habitat is specific for several fungi which will be discussed later. In this environment, the normally saprophytic organisms proliferate and develop. This habitat is also the source of fungal elements and/or spores, where man and animals, incidental hosts, are exposed to the infectious particles. It is important to be aware of these associations to diagnose mycotic diseases. The physician must be able to elicit a complete history from the patient including occupation, avocation and travel history. This information is frequently required to raise, or confirm, your differential diagnosis. The incidence of mycotic infections is currently increasing dramatically, due to an increased population of susceptibles. Examples are patients with AIDS, patients on immunosuppressive therapy, and the use of more invasive diagnostic and surgical procedures (prosthetic implants). Fungal diseases are non-contagious and non-reportable diseases in the national public health statistics.

Diagnosis

1. Skin scrapings suspected to contain dermatophytes or pus from a lesion can be mounted in KOH on a slide and examined directly under the microscope.
2. Skin testing (dermal hypersensitivity) used to be popular as a diagnostic tool, but this use is now discouraged because the skin test may interfere with serological studies, by causing false positive results.

3. Serology may be helpful when it is applied to a specific fungal disease; there are no screening antigens for “fungi” in general. Because fungi are poor antigens, the efficacy of serology varies with different fungal infections. The serologic tests will be discussed under each mycosis. The most common serological tests for fungi are based on latex agglutination, double immunodiffusion, complement fixation and enzyme immunoassays.

4. Direct fluorescent microscopy may be used for identification, even on non-viable cultures or on fixed tissue sections. The reagents for this test are difficult to obtain.

5. Biopsy and histopathology. A biopsy may be very useful for the identification and as a source of the of tissue-invading fungi.

6. Culture. A definitive diagnosis requires a culture and identification. Pathogenic fungi are usually grown on Sabouraud dextrose agar. It has a slightly acidic pH (~5.6); cyclohexamide, penicillin, streptomycin or other inhibitory antibiotics are often added to prevent bacterial contamination and overgrowth. The cultures are examined macroscopically and microscopically. They are not considered negative for growth until after 4 weeks of incubation.

NUTRITION, GROWTH AND ENERGY METABOLISM

PHYSIOLOGY OF MICROORGANISMS includes:

- o Types of microbes nutrition.
- o Types of microbes respiration.
- o Cultivation (environmental conditions, rate and growth character).
- o Biochemical activity.
- o Variability.
- o Formation of BAS, toxins and other factors of pathogenicity.
- o Sensitivity to antibiotics, bacteriophages, bacteriocins.
- o Others biological properties.

Bacterial metabolism is combination of physical and chemical processes providing a living activity of microbial cell. Metabolism consists of two main directions: **anabolism** (biosynthesis of polymeric compounds — proteins, NA, polysaccharides — from monomers) and **catabolism** (biodisintegration of complex polymeric compounds).

Bacterial nutrition

Every organism must find in its environment all of the substances required for energy generation and cellular biosynthesis. The chemicals and elements of this environment that are utilized for bacterial growth are referred to **nutritional requirements**. In the laboratory, bacteria are grown in **culture media** which are designed to provide all the essential nutrients in solution for bacterial growth. For growth and multiplication of bacteria, the minimum nutritional requirements are water, a source of carbon, a source of nitrogen and some inorganic salts. On basis of nutritional types bacteria are divided on four types.

Table 4 — Types of bacterial nutrition

Nutritional type	Energy source	Carbon source	Examples
Photoautotrophs	Light	CO_2	Cyanobacteria, some Purple and Green Bacteria
Photoheterotrophs (photoorganotrophs)	Light	Organic compounds	Some Purple and Green Bacteria
Chemoautotrophs (Lithoautotrophs)	Inorganic compounds, e.g. H_2, NH_3, NO_2, H_2S	CO_2	A few Bacteria and many Archaea
Chemoheterotrophs (chemoorganotrophs)	Organic compounds	Organic compounds	Most Bacteria, some Archaea

On the basis of **carbon** and **energy sources** for growth four major groups of procaryotes may be defined. Bacteria which derive their energy from sunlight are called **phototrophs** and those that obtain energy from chemical reactions are called **chemotrophs**. Bacteria that can synthesise all their organic compounds are called **autotrophs**. Those that are unable to synthesise their own metabolites and depend on preformed organic compounds are called **heterotrophs**. Heterotrophic bacteria that utilize these organic compounds from environment are called **saprophytes** and from living organisms are called **parasites.**

Some bacteria require certain organic compounds in minute quantities because they fulfill specific roles in biosynthesis. There are known as **growth factors**. Growth factors are called “essential” when growth does not occur in their absence or “accessory when they enhance growth. The need for a growth factor results from either a blocked or missing metabolic pathway in the cells.

Growth factors are organized into three categories:

1. **Purines and pyrimidines**: required for synthesis of nucleic acids (DNA and RNA);

2. **Amino acids**: required for the synthesis of proteins;

3. **Vitamins**: needed as coenzymes and functional groups of certain enzymes

Some bacteria (e.g E. coli) do not require any growth factors: they can synthesise all essential purines, pyrimidines, amino acids and vitamins, starting with their carbon source, as part of their own intermediary metabolism. Certain other bacteria (e.g. *Lactobacillus*) require purines, pyrimidines, vitamins and several amino acids in order to grow. These compounds must be added in advance to culture media that are used to grow these bacteria. The growth factors are not metabolized directly as sources of carbon or energy; rather they are assimilated by cells to fulfill their specific role in metabolism. Mutant strains of bacteria that require some growth factor not needed by the wild type (parent) strain are referred to as **auxotrophs**. Thus, a strain of *E. coli* that requires the amino acid tryptophan in order to grow would be called a tryptophan auxotroph and would be designated *E. coli**trp-.*** Thus, bacteria that can make a growth factors’ synthesis are called **prototrophic bacteria**. If cannot do that – **auxotrophic bacteria**.

Bacterial enzymes

Bacterial cells can change patterns of enzymes, in order to adapt them to their specific environment. Often the concentration of an enzyme in a bacterial cell depends on the presence of the substrate for the enzyme. **Constitutive enzymes** are always produced by cells independently of the composition of the medium in which the cells are grown. The enzymes that operate during glycolysis and the TCA cycle are generally constitutive: they are present at more or less the same concentration in cells at all times. **Inducible enzymes** are produced ("turned on") in cells in response to a particular substrate; they are produced only when needed. In the process of induction the substrate, or a compound structurally similar to the substrate, evokes formation of the enzyme and is sometimes called an **inducer**. A **repressible enzyme** is one whose synthesis is down regulated or "turned off" by the presence of (for example) the end product of a pathway that the enzyme normally participates in. In this case, the end product is called a **corepressor** of the enzyme.

Oxygen requirements and metabolism

Oxygen is a universal component of cells and is always provided in large amounts by H_2O. However, prokaryotes display a wide range of responses to molecular oxygen O_2. Depending on the influence of oxygen on growth bacteria are divided into **aerobes** and **anaerobes**.

Aerobic bacteria require oxygen for growth; they use O_2 as a final electron acceptor in **aerobic respiration**. The anaerobes use electron acceptor other than oxygen such as nitrates or sulphates (**anaerobic respiration**). A more common process in anaerobic metabolism may be a series of oxidoreductions in which the carbon and

energy source acts as both the electron donor and electron acceptor. This process is known as **fermentation** and leads to the formation of several organic end products.

Obligate aerobes (like cholera vibrio) which grow only in presence of oxygen.

Obligate anaerobes (like clostridia) do not need or use O_2 as a nutrient. In fact, O_2 is a toxic substance, which either kills or inhibits their growth. Obligate anaerobic prokaryotes may live by fermentation, anaerobic respiration, bacterial photosynthesis, or the novel process of methanogenesis.

Facultative anaerobes (or **facultative aerobes**) are organisms that can switch between aerobic and anaerobic types of metabolism. Under anaerobic conditions (no O_2) they grow by fermentation or anaerobic respiration, but in the presence of O_2 they switch to aerobic respiration. Most bacteria of medical importance are facultative anaerobes!

Aerotolerant anaerobes are bacteria with an exclusively anaerobic (fermentative) type of metabolism but they are insensitive to the presence of O_2. They live by fermentation alone whether or not O_2 is present in their environment.

Microaerophilic bacteria are those that grow best in the presence of a low oxygen concentration.

All bacteria require small amounts of carbon dioxide for growth. Some bacteria (Brucella abortus) require much higher levels of carbon dioxide (5–10%) for growth (**capnophilic**).

The response of an organism to O_2 in its environment depends upon the occurrence and distribution of various enzymes which react with O_2 and various oxygen radicals that are invariably generated by cells in the presence of O_2. All cells contain enzymes capable of reacting with O_2. For example, oxidations of flavoproteins by O_2 invariably result in the formation of H_2O_2 (peroxide) as one major product and small quantities of an even more toxic free radical, superoxide or $O_2^{\cdot-}$. Also, chlorophyll and other pigments in cells can react with O_2 in the presence of light and generate singlet oxygen, another radical form of oxygen which is a potent oxidizing agent in biological systems.

In aerobes and aerotolerant anaerobes the potential for lethal accumulation of superoxide is prevented by the enzyme **superoxide dismutase**. All organisms which can live in the presence of O_2 contain superoxide dismutase. Nearly all organisms contain the enzyme **catalase**, which decomposes H_2O_2. Even though certain aerotolerant bacteria such as the lactic acid bacteria lack catalase, they decompose H_2O_2 by means of **peroxidase** enzymes which derive electrons from $NADH_2$ to reduce peroxide to H_2O. Obligate anaerobes lack superoxide dismutase and catalase and/or peroxidase, and therefore undergo lethal oxidations by various oxygen radicals when they are exposed to O_2. All photosynthetic organisms are protected from lethal oxidations of singlet oxygen by their possession of **carotenoid pigments** which physically react with the singlet oxygen radical and lower it to its nontoxic "ground" (triplet) state.

Bacterial reproduction and growth

Ways of reproduction in bacteria:

1. **Binary division** (most of bacteria).
2. **Budding** (Mycoplasmas).
3. Filamentary forms **segmenting** (Actinomycetes).
4. With help of the **exospores** (Streptomycetes).
5. **Particular cycle of division** (Chlamydia).

Growth is an orderly increase in the quantity of cellular constituents. It depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. In most bacteria, growth involves increase in cell mass and number of ribosomes, duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of the two chromosomes, septum formation, and cell division. This asexual process of reproduction is called **binary fission**. For unicellular organisms such as the bacteria, growth can be measured in terms of two different parameters: changes in **cell mass** and changes in **cell numbers**.

Bacterial colonies growing on a plate of nutrient agar in the laboratory, under favorable conditions, a growing bacterial population doubles at regular intervals. Growth is by geometric progression: 1, 2, 4, 8, etc. or 2^0, 2^1, 2^2, 2^3.....2^n (where n = the number of generations). This is called **exponential growth**. In reality, exponential growth is only part of the bacterial life cycle, and not representative of the normal pattern of growth of bacteria in nature.

When a fresh medium is inoculated with a given number of cells, and the population growth is monitored over a period of time, plotting the data will yield a **typical bacterial growth curve (see figure 26)**

The curve shows the fallowing phases:

1. **Lag Phase**: Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity. The length of the lag phase is apparently dependent on a wide variety of factors including the size of the inoculum; time necessary to recover from physical damage or shock in the transfer; time required for synthesis of essential coenzymes or division factors; and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium.

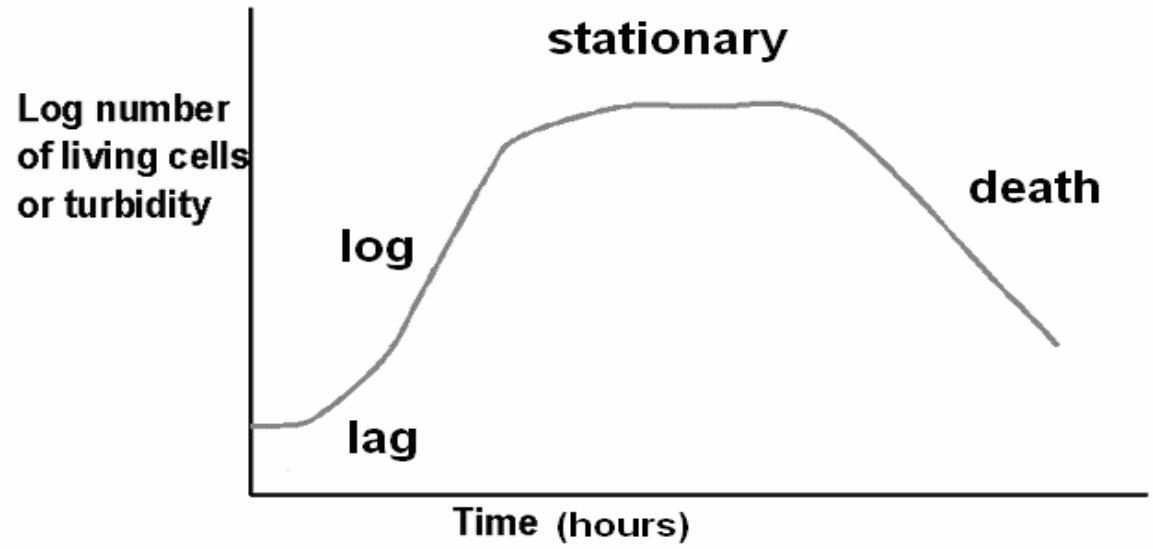


Figure 26 — Bacterial growth curve

2. **Log (logarithmic) or exponential phase**: The exponential phase of growth is a pattern of balanced growth wherein all the cells are dividing regularly by binary fission, and are growing by geometric progression. The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incuba-

tion. The rate of exponential growth of a bacterial culture is expressed as **generation time**, also the **doubling time** of the bacterial population. Generation time (G) is defined as the time (t) per generation (n = number of generations). Hence, G=t/n is the equation from which calculations of generation time derive.

3. **Stationary Phase**. Exponential growth cannot be continued forever in a **batch culture** (e.g. a closed system such as a test tube or flask). Population growth is limited by one of **three factors:** 1) exhaustion of available nutrients; 2) accumulation of inhibitory metabolites or end products; 3) exhaustion of space, in this case called a lack of "biological space". The number of progeny cells formed is just enough to replace the number of cells that die. The viable count remains stationary as equilibrium exists between the dying cells and the newly formed cells. The stationary phase, like the lag phase, is not necessarily a period of quiescence. Bacteria that produce **secondary metabolites**, such as antibiotics or exotoxins, do so during the stationary phase of the growth cycle (secondary metabolites are defined as metabolites produced after the active stage of growth). Sporulation occurs at this stage and bacteria frequently are Gram variable.

4. **Death Phase**. If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. (Note, if counting by turbidimetric measurements or microscopic counts, the death phase cannot be observed.). During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.

Physical and environmental requirements for microbial growth

The effect of pH on growth: The pH, or hydrogen ion concentration, $[H^+]$, of natural environments varies from about 0.5 in the most acidic soils to about 10.5 in the most alkaline lakes. The range of pH over which an organism grows is defined by **three cardinal points**: the **minimum pH**, below which the organism cannot grow, the **maximum pH**, above which the organism cannot grow, and the **optimum pH**, at which the organism grows best. For most bacteria there is an orderly increase in growth rate between the minimum and the optimum and a corresponding orderly decrease in growth rate between the optimum and the maximum pH, reflecting the general effect of changing $[H^+]$ on the rates of enzymatic reaction.

Microorganisms which grow at an optimum pH well below neutrality (7.0) are called **acidophiles (**such as lactobacilli). Those which grow best at neutral pH are called **neutrophiles** and those that grow best under alkaline conditions are called **alkaliphiles** (cholera vibrio). The majority of pathogenic bacteria grow best at neutral or slightly alkaline reaction (pH 7.2–7.6). Strong solutions of acid or alkali (5% HCl or NaCl) readily kill most bacteria, though mycobacteria are exceptionally resistant to them.

The effect of temperature on growth: A particular microorganism will exhibit a range of temperature over which it can grow, defined by three cardinal points in the same manner as pH. Considering the total span of temperature where liquid water exists, the prokaryotes may be divided into several subclasses on the basis of one or another of their cardinal points for growth. For example, organisms with an optimum temperature near 37 degrees (the body temperature of warm-blooded animals) are called **mesophiles**. Organisms with an optimum T between about 45 degrees and 70 degrees are **thermophiles**. Some Archaea with an optimum T of 80 degrees or higher and a maximum T as high as 115 degrees, are now referred to as **extreme thermophiles** or **hyperthermophiles**. The cold-loving organisms are **psychrophiles** defined by their ability to grow at 0 degrees. A variant of a psychrophile (which usually has an optimum T

of 10–15 degrees) is a **psychrotrophiles**, which grows at 0 degrees but displays an optimum T in the mesophile range, nearer room temperature. Psychrotrophs are the scourge of food storage in refrigerators since they are invariably brought in from their mesophilic habitats and continue to grow in the refrigerated environment where they spoil the food. Of course, they grow slower at 2 degrees than at 25 degrees. Think how fast milk spoils on the counter top versus in the refrigerator.

Water availability: Water is the solvent in which the molecules of life are dissolved, and the availability of water is therefore a critical factor that affects the growth of all cells. The only common solute in nature that occurs over a wide concentration range is salt [NaCl], and some microorganisms are named based on their growth response to salt. Microorganisms that require some NaCl for growth are **halophiles**. **Mild halophiles** require 1–6% salt, **moderate halophiles** require 6–15% salt; **extreme halophiles** that require 15–30% NaCl for growth are found among the archaea. Bacteria that are able to grow at moderate salt concentrations, even though they grow best in the absence of NaCl, are called **halotolerant**. Although halophiles are "osmophiles" (and halotolerant organisms are "osmotolerant") the term **osmophiles** is usually reserved for organisms that are able to live in environments high in sugar. Organisms which live in dry environments (made dry by lack of water) are called **xerophiles**.

Culturing of the microorganisms

The cultures so far discussed for growth of bacterial populations are called **batch cultures**. Since the nutrients are not renewed, exponential growth is limited to a few generations. Bacterial cultures can be maintained in a state of exponential growth over long periods of time using a system of **continuous culture**, designed to relieve the conditions that stop exponential growth in batch cultures. Continuous culture, in a device called a **chemostat**, can be used to maintain a bacterial population at a constant density, a situation that is, in many ways, more similar to bacterial growth in natural environments. Studying the growth of bacterial populations in batch or continuous cultures does not permit any conclusions about the growth behavior of individual cells, because the distribution of cell size (and hence cell age) among the members of the population is completely random. Information about the growth behavior of individual bacteria can, however, is obtained by the study of **synchronous cultures**. Synchronized cultures must be composed of cells which are all at the same stage of the **bacterial cell cycle**. Measurements made on synchronized cultures are equivalent to measurements made on individual cells.

Culture media for the growth of bacteria

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment. The biochemical (nutritional) environment is made available as a **culture medium**, and depending upon the special needs of particular bacteria (as well as particular investigators) a large variety and types of culture media have been developed with different purposes and uses. Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties.

Media have been classified in many ways

1. Liquid media are used for growth of pure batch cultures, while **solid** media are used widely for the isolation of pure cultures, for estimating viable bacterial populations, and a variety of other purposes. The usual gelling agent for **solid** or **semi-**

solid medium is *agar*, a hydrocolloid derived from red algae. Agar is used because of its unique physical properties (it melts at 100 degrees and remains liquid until cooled to 40 degrees, the temperature at which it gels) and because it cannot be metabolized by most bacteria. Hence as a medium component it is relatively inert; it simply holds (gels) nutrients that are in aqueous solution.

2. Simple (basal) media such as nutrient agar (MPA) and nutrient broth (MPB) that will support the growth of microorganisms that do not have special nutrient requirements. Nutrient broth consists of peptone, meat extract, NaCl and water. Nutrient agar, made by adding 2% agar to nutrient broth, is the simplest and most common medium for diagnostics laboratories. If the concentration of agar is reduced to 0.5%, semisolid agar is obtained which enables motile bacteria to spread. **Complex** media have added ingredients for special purposes or providing special nutrients required for the growth of bacterium under study.

3. Culture media may be classified into several categories depending on their composition. A **chemically-defined (synthetic)** media is one in which the exact chemical composition is known, are prepared by mixing pure chemical substance (salts, as a rule). A **complex (undefined)** media is one in which the exact chemical constitution of the medium is not known. Defined media are usually composed of pure biochemicals; complex media usually contain complex materials of biological origin such as blood or milk or yeast extract or beef extract, the exact chemical composition of which is obviously undetermined. A defined medium is a **minimal medium** if it provides only the exact nutrients (including any growth factors) needed by the organism for growth. The use of defined minimal media requires the investigator to know the exact nutritional requirements of the organisms in question. Most pathogenic bacteria of animals, which have adapted themselves to growth in animal tissues, require complex media for their growth. Blood, serum and tissue extracts are frequently added to culture media for the cultivation of pathogens. Even so, for a few fastidious pathogens such as *Treponema pallidum*, the agent of syphilis, and *Mycobacterium leprae*, the cause of leprosy, artificial culture media and conditions have not been established.

4. Special media are divisible into: **Enriched** media with substances such as blood, serum, egg are added to a simple medium. They are used to grow bacteria which are more exacting in their nutritional needs (e.g. blood agar and egg media). This is useful when the organism you wish to culture is present in relatively small numbers compared to the other organisms growing in the mixture.

Selective media which contain substances that prevent the growth of microorganisms other than the pathogens for which the media are intended. So selective medium has agents added which will **inhibit the growth of one group of organisms while permitting the growth of another**. The inhibitory substance may be salt (NaCl), acid, a toxic chemical (crystal violet), an antibiotic (streptomycin), or some other substance. For example, **Columbia CNA agar** has the antibiotic nalidixic acid added which inhibit the growth of gram-negative bacteria but not the growth of gram-positives.

Differential (indicator) media are media to which indicators, dyes or other substances are added to differentiate microorganisms. Most, but not all differential media distinguish between bacteria by an indicator which changes color when acid is produced following carbohydrate fermentation. They are useful in differentiating bacteria according to some biochemical characteristic. In other words, *they indicate whether or not a certain organism can carry out a specific biochemical reaction* during its normal metabolism. In some cases media have been formulated that are both selective and differential. A good example is Levine EMB agar, which is used to determine the presence of coliforms in water analysis.

Also example: *eosin-methylene blue agar* and *Mac-Conkey agar* contains lactose and dye or an indicator in the decolourized state. Bacteria which ferment lactose with the production of acid will produce red colour of colonies with metallic sheen. So in practice you may differentiate colonies of the lactose fermenting bacteria from colonies of lactose non-fermenting organisms.

Transport media are mostly semisolid media that contain ingredients to prevent the overgrowth of commensals and ensure the survival of pathogens when specimens can not be cultured soon after collection.

Characteristics of bacterial growth in nutrient media

Liquid nutrient media:

- **Diffuse suspension** (the most of bacteria)
- **Film** («Koch's bacteria»)
- **Near-bottom** or **near-wall growth** (streptococci)
- Film on the surface with **thread-like growth** resembling stalactites and a flocculent precipitate (*Yersinia pestis*)

Solid nutrient media (formation of **colonies**, which are clones of cells originating from a single bacterial cell):

- **S-form** or «smooth» colony (cocci and Gram-negative rods, except *Yersinia pestis*)
- **R-form** or «rough» colony (Gram-positive rods and *Yersinia pestis*).

Microbial growth requirements

Before one can construct a medium that will achieve a desired result in the growth of organisms, one must understand their basic needs. Any medium that is to be suitable for a specific group of organisms must take into account the following seven factors: **water, carbon, energy, nitrogen, minerals, growth factors and pH.** Other substances may be also being included depending on the specific requirements of individual pathogen.

It has been estimated that 80% of the living weight of a bacterial cell is water, and of the dry weight, 2–5% is phosphorus and the remainder is made up of various minerals and combinations of oxygen and hydrogen in organic compounds.

Although autotrophic organisms can utilize inorganic sources of nitrogen, the heterotrophs get their nitrogen from amino acids and intermediate protein compounds such as peptides and peptones. Beef extract and peptone, as used in nutrient broth; provide the nitrogen needs for the heterotrophs grown in this medium.

All organisms require several metallic elements such as sodium, potassium, calcium, magnesium, manganese, iron, zinc, copper, phosphorus, and cobalt for normal growth. The amounts required are very small.

The growth of organisms in a particular medium may be completely inhibited if the pH of the medium is not within certain limits. The enzymes of microorganisms are greatly affected by this factor. Since most bacteria grow best around pH 7 or slightly lower, the pH of nutrient broth should be adjusted to pH 6.8. Most bacteria, pathogenic in humans, give optimum growth when incubated at body

temperature, i.e., 37°C. Some saprophytes however, grow best at lower temperatures, even as low as 4°C and others at high temperatures. The latter are known as thermophilic bacteria and are used in testing effectiveness of sterilization techniques. Most organisms need oxygen for growth and are incubated in normal atmospheric conditions. Some pathogens, e.g., tetanus bacilli, will grow only in the absence of oxygen.

Cultural features of bacteria

- Nutrients needs
- Optimal nutrient medium
- Temperature conditions
- Aeration conditions
- Rate of growth
- Characteristics of growth on liquid and solid nutrient media.

Culture methods

When we try to study the bacterial flora of the body, soil, water, food, or any other part of our environment, we soon discover that bacteria exist in mixed populations. It is only in very rare situations that they occur as a single species. To be able to study the cultural, morphological, and physiological characteristics of an individual species, it is essential, first of all, that the organism be separated from the other species that are normally found in its habitat; in other words, we must have a **pure culture** of the microorganism.

Several different methods of getting a pure culture from a mixed culture are available to us. The two most frequently used methods involve making a **streak plate** or a **pour plate (loop dilution)**. Both plate techniques involve thinning the organisms so that the individual species can be selected from the others. There are also known such methods **lawn, stroke, stab and liquid cultures**. **Special methods** are employed for culturing anaerobic bacteria. For estimating bacteria in the dust on clothing, the **sweep plate** method is used.

STREAK PLATE METHOD: For economy of materials and time, this method is best. This method is employed for the isolation of bacteria in pure culture from clinical specimens (see figure 28). Figure 27 illustrates how colonies of a mixed culture should be spread out on a properly made streak plate. The important thing is to produce good spacing between colonies.

Figure 27 — Different streak techniques

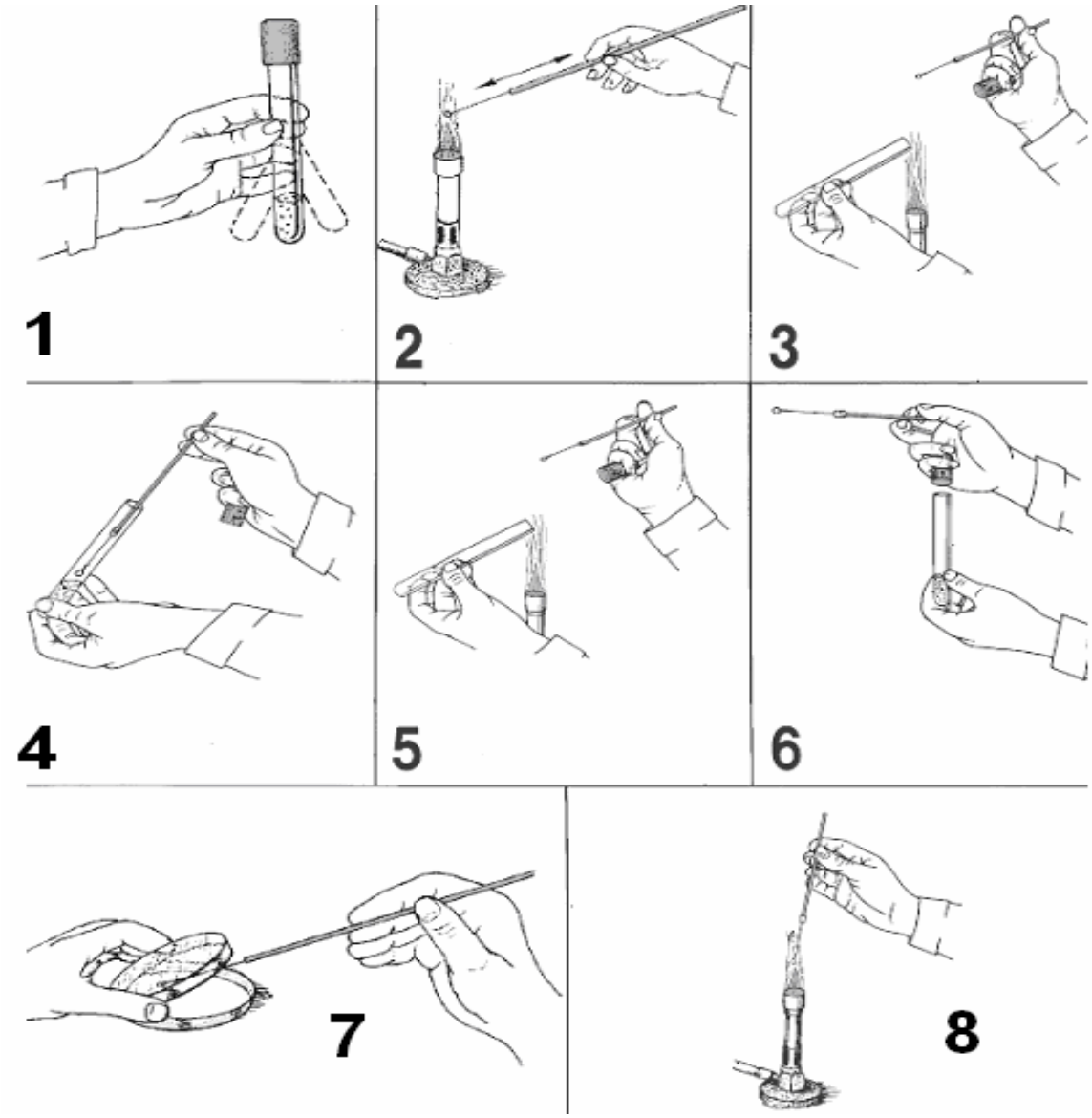


Figure 28 — Streak plate method:

1. Shake the culture tube side to side to suspend organisms. 2. Heat the loop and wire to red-hot. 3. Remove the cap and flame the top of the tube. Do not place the cap down on the table. 4. After allowing the loop to cool for at least 5 seconds, remove a loopful of organisms. Avoid touching the sides of the tube! 5. Flame the top of tube again. 6. Return the cap to the tube. 7. Streak the plate, holding it as shown. Do not gouge into the medium with the loop. 8. Flame the loop before placing it down.

POUR PLATE METHOD (Loop dilution): This method of separating one species of bacteria from another consists of diluting out one loopful of organisms with three tubes of liquid nutrient agar in such a manner that one of the plates poured will have an optimum number of organisms to provide good isolation. Figure 29 illustrates the general procedure. One advantage of this method is that it requires somewhat less skill than that required for a good streak plate; a disadvantage, however, is that it requires more media, tubes and plates. The pour plate method gives an estimate of the viable bacteria count in a suspension and is the recommended method for quantitative urine cultures.

The **lawn or carpet** culture provides a uniform surface growth of the bacterium and is useful for bacteriophages typing and antibiotic sensitivity testing. Lawn cultures are prepared by flooding the surface of the plate with a liquid culture of the bacterium.

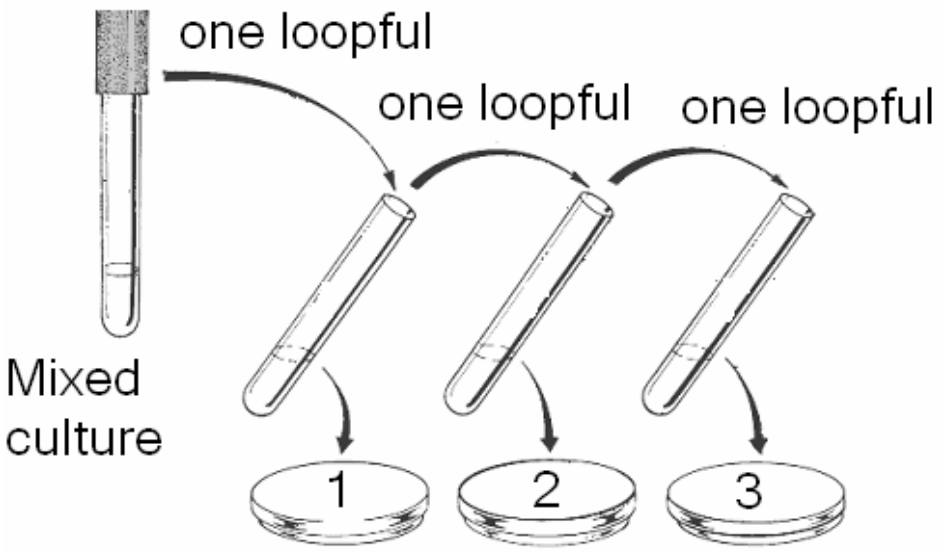


Figure 29 — Three steps in the loop dilution technique for separating out organisms

The **stroke culture** is made in tubes containing agar slope (slant) and is employed for providing a pure growth of the bacterium for different diagnostic tests.

Stab cultures are prepared by puncturing a suitable medium (e.g. gelatin agar) with a long wire (needle). The medium is allowed to set in the vertical position, providing a flat surface at the top of the medium. Stab cultures are employed for demonstration of *gelatin liquefaction* (fluidifying) and oxygen requirements of the bacterium under the study.

In **sweep plate method**, the edges of the Petri dishes containing the culture medium are rubbed over the fabric, with the medium facing it. The dust particles stirred up from the cloth settle on the culture medium and colonies develop on incubation. They can be counted and estimates made.

Liquid cultures in tubes may be inoculated by touching with loop or by adding the inoculum with pipettes. This method is adopted for blood culture and for sterility tests where the concentration of bacteria in the inocula are expected to be small. The disadvantage is that it does not provide a pure culture from mixed inocula.

Culturing of anaerobes

The above-stated procedures for culturing bacteria that were used well only if the organisms will grow in the presence of oxygen. Unfortunately, there are many bacteria that find oxygen toxic or at least inhibitory to their existence (*Clostridium species, Bacteroides species and anaerobic streptococci*).

There are several techniques for obtaining anaerobic conditions:

● **Anaerobic jar with hydrogen** from a cylinder.

To provide an oxygen-free incubation environment for the Petri plates of anaerobic agar we will use the **GasPak anaerobic jar.** Note in hydrogen is generated in the jar, which removes the oxygen by forming water. Palladium pellets catalyze the reaction at room temperature. The generation of hydrogen is achieved by adding water to a plastic

envelope of chemicals. To make certain that anaerobic conditions actually exist in the jar, an indicator strip of methylene blue becomes colorless in the total absence of oxygen.

● **Anaerobic jar** (**anaerostat**) with a gas generating kit (displacement of oxygen with gases such as nitrogen, helium or carbon dioxide). For example, candle jar.

● **Copper coated steel wool** to remove oxygen (the metallic copper on the surface of the iron rapidly adsorbs oxygen).

● **Sodium dithionite agar** (contained in a clear agar medium in the lid of Petri dish) to remove oxygen.

● **Reducing agents** in culture media. Examples of media that contain these agents: fluid thioglycollate medium (FTM), tryptone glucose yeast agar (TGYA), and Brewer's anaerobic agar. Each medium will serve a different purpose.

TGYA: The medium is not primarily an anaerobic medium; instead it is a rich general purpose medium that favors the growth of broad spectrum of organisms. It will be inoculated in the liquefied state, shaken to mix the organisms throughout the medium and allowed to solidify. After incubation one determines the oxygen requirements on the basis of where the growth occurs in the tube: top, middle, or bottom. Figure 30 illustrates where anaerobic various types of bacteria tend to grow with respect to the degree of oxygen tension in a medium.

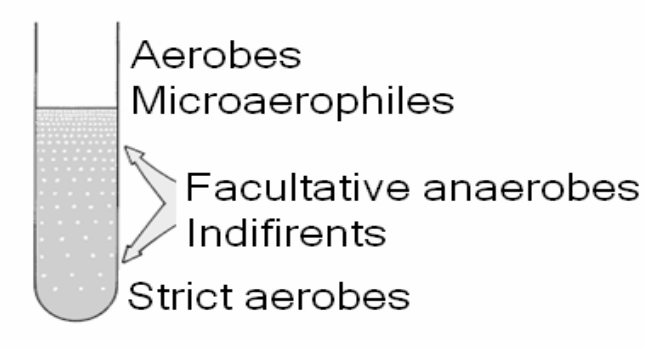


Figure 30 — Sites of growth on the basic oxigen requirement

FTM: medium is a rich liquid medium that supports the growth of both aerobic and anaerobic bacteria. It contains glucose, cystine and *sodium thioglycollate* to reduce its oxidation-reduction (O/R) potential. It also contains the dye methylene blue that is an indicator for the presence of oxygen. In the presence of oxygen the dye becomes pink.

Pobertson' cooked meat medium (***Kitt-Tarocci's with pieces of liver and glucose*** medium also) is the most widely used fluid medium for culture anaerobes. It consists of *fat-free cooked meat* in broth, with a layer of sterile vaseline over it. It permits the growth of even obligate anaerobes and indicates their saccharolytic or proteolytic activities (meat being turned red or black, respectively).

● Method where **alkaline pyrogallol** absorbs oxygen (Aristovsky's device). Pirogalic acid added to a solution of sodium hydroxide in a large test tube placed inside an airtight jar provides anaerobiosis but a small amount of CO, which is formed during the reaction, may be inhibitory to some bacteria.

● **Boil** a tube of nutrient broth and layer over it sterile **vaseline**. The boiling removes the oxygen and the vaseline prevents more entering as the broth cools. The tube is inoculated using a sterile Pasteur pipette.

● **Combined cultivation** of aerobes with anaerobes on the same Petri dish (Forthner's method).

Identification of unknown bacteria

Microbiological (bacteriological) method of investigation is based on the isolation of a pure culture of microbe and it identification.

In order to study the properties of given microorganism, it is necessary to receive pure culture of this microbe is free from all other kinds of organisms.

Advantages of this method: respectively high sensitivity and accuracy; ability to detect the exact quantity of microbes in the investigated material and sensitivity to the antibiotics also. **Disadvantages:** expensive and long-term method.

Methods for isolating pure culture

1. **Surface plating** is the method employed in clinical bacteriology and enables to isolation of distinct colonies.
2. **Enriched, selective and differential media** are widely used for the isolation pathogens from specimens such as feces with varied flora.
3. **Pretreatment of specimens with bactericidal substances** which destroy the unwanted bacteria (e.g. sputum is treated by alkali or acid, after that most of sensitive bacteria are killed but tubercle bacilli are resistant).
4. *Obligate aerobes and anaerobes* may be separated by cultivation under aerobic or anaerobic conditions.
5. Separation of bacteria with **different temperature optima** can be effected by incubation at different temperatures.
6. By heating a mixture containing vegetative and spore forming bacteria, at 80ºC the vegetative forms can be eliminated.
7. Separation of *motile from nonmotile bacteria*.
8. Pathogenic bacteria may be isolated by inoculation into special animals.
9. Bacteria of different sizes may be separated by the use of selective filters (e.g. separating viruses from bacteria).

Stages of bacteriological investigation

1. The taking a **pathological material**
2. Process of **OBTAINING A PURE CULTURE** of microbe

The *first day* of investigation*:*

- **Preliminary microscopy** of the investigated material (aim: *to detect approximate composition of the material*).
- **Inoculation** of the pathological material on a suitable type of **nutritional medium** depending on the purpose of given investigation (aim: *to receive isolated colonies in order to separate different types of bacteria from material mixture*).

The *second day* of investigation*:*

- Study of **cultural characteristics of colonies** (aim: *to get additional information for the identification of the bacterium*).
- **Re-inoculation** of colonies on a slant agar (aim: *to receive and accumulate a pure culture of the bacterium*).

3. Process of **IDENTIFICATION of microbe pure culture**

The *third day* of investigation:

- Check of cleanliness (purity) of isolated pure culture.
- Study of fermentation and other biochemical properties of the bacterium.
- Estimation of bacterial antigenic structure.
- Study of bacterium sensitivity to antibiotics, bacteriophages, bacteriocin typing,
- Estimation of pathogenicity and virulence

Aim of identification: *species and subspecies (infraspecific variant) determination of investigated bacterium*.

4. **Conclusion** (species of given microbe is established after a comparison with the appropriate properties of standard specific strain).

Obtaining of anaerobic pure culture

The first day of investigation: **Preliminary microscopy** of the investigated material and **inoculation** on the *special liquid medium of accumulation for anaerobic bacteria* (**Kitt-Tarocci's medium**), covering by vaseline and placement in the thermostat.

The second day of investigation: **Re-inoculation** of grown turbid medium from the tube on the fresh solid medium (*blood-sugar agar*) or on the *stab sugar agar* (then in thermostat on 1–2 days). Aim: to receive isolated colonies of bacteria.

The third day of investigation: **Study of colonies** (morphological, biochemical, cultural properties) and **repeated inoculation** on Kitt-Tarocci's medium for accumulation of a pure culture of anaerobic microbes.

Morphology and staining reactions

The **first step** in the identification of an unknown bacterial organism is to learn as much as possible about its **morphological characteristics**. The characteristics noted are **shape, size, arrangement, motility, flagella, spores and capsules**.

The age of the culture is important. In older cultures, staining characteristics vary well. GRAM STAIN divides bacteria into Gram positive and Gram negative. SIMPLE STAIN brings out the morphology of bacteria best. ACID-FAST STAINING (Ziehl-Neelsen staining) stains into acid fast and non-acid fast bacteria. Differential and special stains are necessary to bring out characteristics such as flagella, capsules, spores and storage granules. The fluorescent antibody technique enables to identify bacteria according to their surface antigens. MOTILITY TESTS: If organism is a nonpathogenic make a wet mount (hanging drop slide) for non-pathogens or SEMISOLID MEDIUM for pathogens.

Cultural characteristics

The cultural characteristics of an organism pertain to its macroscopic appearance on different kinds of media (*character of microbial growth on the solid and liquid media*). **Cultural properties are important diagnostic feature of each species of microorganisms!** The most frequently used media for a cultural study are nutrient agar, nutrient broth, and nutrient gelatin. For certain types of unknowns it is also desirable to inoculate a blood agar plate. In addition to these media, you will be inoculating a fluid thioglycollate medium to determine the oxygen requirements.

While studying colonies on solid media, these ones are described (see figure 31) **as:**

◦ **Shape:** Circular, irregular or rhizoid.
◦ **Surface:** Smooth, rough, wavy, granular, papillate etc.
◦ **Size:** Usually colonies are 2–3 mm; smaller ones may be less than 1 mm.
◦ **Contiguity:** Colonies may be discrete or swarming.
◦ **Consistency:** May be mucoid, viscous, dry or adherent to the medium.
◦ **Pigmentation:** Some organisms produce pigmented colonies (Staphylococci).
◦ **Opacity:** On nutrient agar they may be transparent, translucent or opaque.
◦ **Elevation:** Colonies may be effuse, elevated, convex, concave, umbilicate etc.
◦ **Edges**: entire, undulate, fimbriate or curled.
◦ **Amount of growth:** The abundance of growth may be described as none, slight, moderate, and abundant.
◦ **Emulsifiability** and whether they are differentiated into a central and peripheral portion.
◦ **Color:** Pigmentation should be looked for on the organisms and within the medium. Most organisms will lack chromogenesis, exhibiting a white growth; others are various shades of different colors. Some bacteria produce soluble pigments that diffuse into the medium. Hold the slant up to a strong light to examine it for diffused pigmentation/

In a **fluid medium**, the degree of growth, presence of turbidity and its nature, presence of deposit and its character, nature of surface growth and its quantity.

Surface: Figure illustrates different types of surface growth. Pellicle type of surface differs from the membranous type in that the latter is much thinner. A *flocculent* surface is made up of floating adherent masses of bacteria.

Sediment The amount of sediment in the bottom of the tube may vary from none to a great deal. To describe the type of sediment, agitate the tube, putting the material in suspension. The type of sediment can be described as *granular, flocculent, flaky,* and *viscid.* Test for viscosity by probing the bottom of the tube with a sterile inoculating loop.

Amount of growth To determine the amount of growth, it is necessary to shake the tube to disperse the organisms. Terms such as *slight* (scanty), *moderate,* and *abundant adequately* describe the amount.

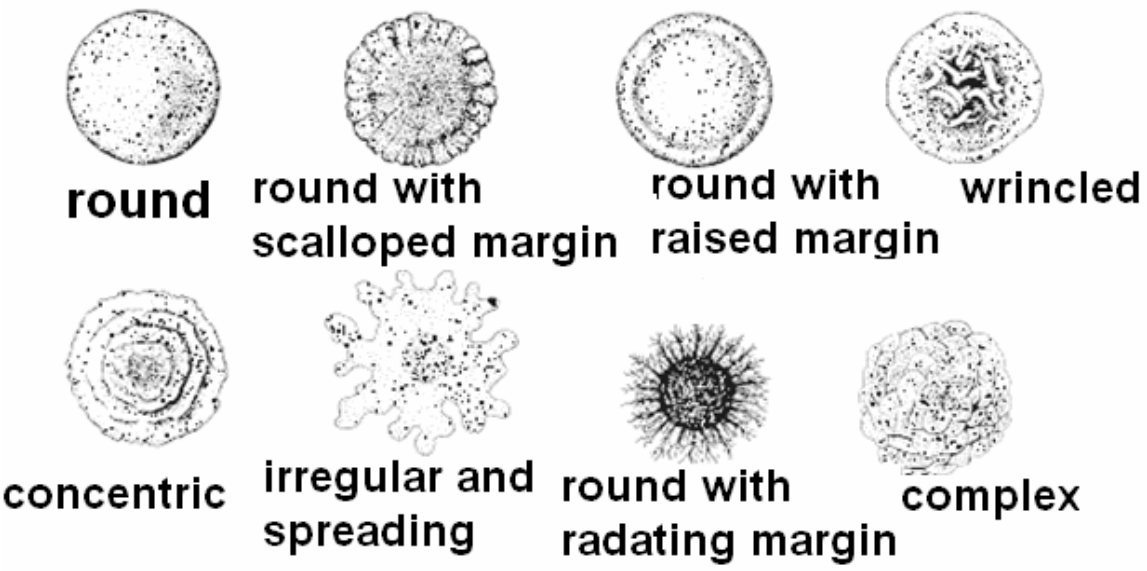
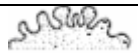
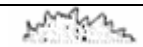


Figure 31 — Types of microbial colonies

Table 5 — Cultural characteristics

Cultural characteristic	Image	Cultural characteristic	Image
Flat		Smooth	
Raised		Wavy	
Convex		Lobate	
Drop-like		Irregular (rough)	
		Ciliate	
		Branching	
Ingrowing into medium		Thread-like	
Crateriform		“Hair-lock”-like	

Physiological (biochemical) characteristics

Although morphological and cultural characteristics are essential in getting to the genus, species determination requires a good deal more information. The physiological information that will be accumulated here and will make species identification possible.

The chemical reactions that occur within the cells of all living organisms are referred to as **metabolism.** These reactions are catalyzed by protein molecules called **enzymes.** The majority of enzymes function within the cell and are called **endoenzymes.** Many bacteria also produce **exoenzymes,** which are released by the cell to catalyze reactions outside of the cell. In deriving energy from food, bacteria may be either oxidative or fermentative. **Oxidative** bacteria utilize oxygen to yield carbon dioxide and water. These bacteria have a *cytochrome enzyme system.* By utilizing organic compounds as electron donors, with oxygen as the ultimate electron (and hydrogen) acceptor, they produce CO_2 and water as end products. **Fermentative** bacteria, on the other hand, also utilize organic compounds for energy, but they lack a cytochrome system. Instead of producing only CO_2 and water, they produce complex end products, such as acids, aldehydes, and alcohols. Various gases, such as carbon dioxide, hydrogen, and methane, are also produced. In fermentative bacteria, the organic compounds act both as electron donors and electron acceptors.

Each species of microbe produces the *constantly inherent* set of different enzymes for distinctive substrates. The more important and widely used fermentative properties are: 1) Ability to fermentate **carbohydrates** (sugars) with formation of different acids and gases (presence *saccharolytic enzymes*); 2) Ability to decompose the **proteins** (presence *proteolytic enzymes*); 3) Ability to produce an enzyme — *catalase*; 4) Ability to produce other specific enzymes (of invasiveness, aggressiveness and other toxic enzymes).

Determination of sugar fermentation

When we use a set of tubes containing various sugars, we are able to determine what sugars an organism is able to ferment. If an organism is able to ferment a particular sugar, acid will be produced and gas *may* be produced. The presence of acid is detectable with the color change of a pH indicator in the medium. The sugar broths used here contain 0.5% of the specific carbohydrate plus sufficient amounts of beef extract and peptone to satisfy the nitrogen and mineral needs of most bacteria. Although there are many sugars that one might use, glucose, lactose, and mannitol are logical ones to begin with.

For example, **"Motley" Hiss row** (with lactose, glucose, mannitol, maltose, saccharose) is using for family Enterobacteriaceae detection. The principle of operation: fermentation of a sugar is caused by the suitable enzyme occurs. This process leads to change of medium colour due to indicator and appearance of gas bubbles in the test tube.

Also special **differential diagnostic media** (*identification of closely related species of microbes distinguished by biochemical properties*) are employed for selection of different species of Enterobacteriaceae:

- **Endo** (lactose and indicator fuchsine).
- **Levine** (lactose, eosin and methylene blue).
- **Ploskirev** (lactose and neutral red).

Methyl red test (to identify *Enterobacteria*): Organism fermentates glucose, producing acidity in a buffered medium to give a colour change of indicator.

Determination of proteins decomposition

Litmus milk (to identify *enterococci*): There are may be no change in the medium or acid, alkali may be produced; clotting of milk may occur with decolourization of the litmus. Also **milk agar** in the Petri dish is used. Positive result is appearance of turbidity around the colonies (coagulation of milk proteins).

Gelatin Stab Culture: Organisms that are able to liquefy gelatin produce the enzyme *gelatinase.* Different types of microbes give a characteristic form of liquation for them as this is illustrated in figure 32.

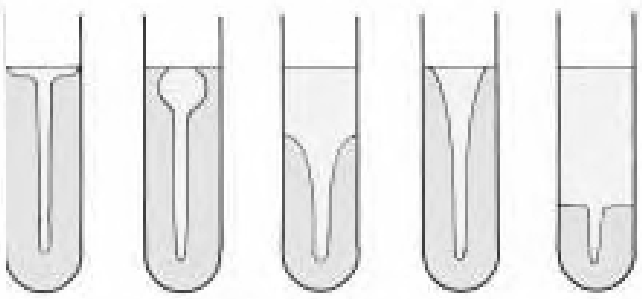


Figure 32 Growth in gelatin stabs

The next tests are shown a dipper decomposition of protein with help of bacterial enzymes.

Indole production (to identify Gram negative rods): this test demonstrates the production of indole from tryptophane. Indicator filter paper is impregnated by solution of oxalic acid. A red colour indicates a positive reaction.

Production of ammonia: this test demonstrates the production of ammonia from aminoacids. Positive result is blue discoloration of the litmus filter paper placed above the culture of given microbe and indicates NH_3 production.

Hydrogen sulphide production: Some bacteria decompose sulphur-containing aminoacids producing H_2S among the products. Indicator filter paper is impregnated by lead acetate. Darkening of the paper indicates H_2S production.

Determination of catalase, oxidase and toxic enzymes

Most aerobes and facultative bacteria that utilize oxygen produce hydrogen peroxide, which is toxic to their own enzyme systems. Their survival in the presence of this antimetabolite is possible because they produce an enzyme called *catalase,* which converts the hydrogen peroxide to water and oxygen: $\mathbf{H_2O_2 \rightarrow H_2 + O_2}$

It has been postulated that the death of strict anaerobes in the presence of oxygen may be due to the suicidal act of H_2O_2 production in the absence of catalase production. The presence or absence of catalase production is an important means of differentiation between certain groups of bacteria. Outer effect in positive test: gas-formation with mixing the culture and hydrogen peroxide.

Hemolysins are enzymes that destroy an erythrocytes membrane (blood agar is used and positive result is hemolysis — light zones — around the colonies). *Plasmocoagulase* is enzyme that coagulates the animal blood (appearance of blood clotting after incubation of the blood and bacterial culture).

Oxidase Production: The production of oxidase is one of the most significant tests we have for differentiating certain groups of bacteria. For example, all the Enterobacteriaceae are oxidase-negative and most species of *Pseudomonas* are oxidase-positive. *Filter Paper Method***:** On a piece of filter paper in a Petri dish, place several drops of oxidase test reagent. Remove a loopful of the organisms from one of the colonies and smear the organisms over a small area of the paper. The positive color reaction described above will show up within 10–15 seconds.

Determination of antigenic structure, pathogenicity and resistance of bacteria to different factors

By using specific serum may by identify bacteria by agglutination and other serological reactions. Immunofluorescence test is useful in some cases.

Pathogenicity tests by inoculation of the bacteria into laboratory animals like guinea pig, rabbit, rat and mouse by intradermal, subcutaneous, intramuscular, intraperitoneal, intracerebral, intravenous or by oral spray were common procedure.

The resistance of the bacteria to heat and to disinfectants is tested, both for vegetative and spore form. **Resistance to antibiotics** (see theme “Antibiotics”) and chemotherapeutic agents and bacteriocins would also help in differentiation and identification of unknown bacterium.

Miniaturized multitest systems

Miniaturized systems have the following advantages over the macromethods:

1. Minimum media preparation;
2. Simplicity of performance;
3. Reliability;
4. Rapid results;
5. Uniform results.

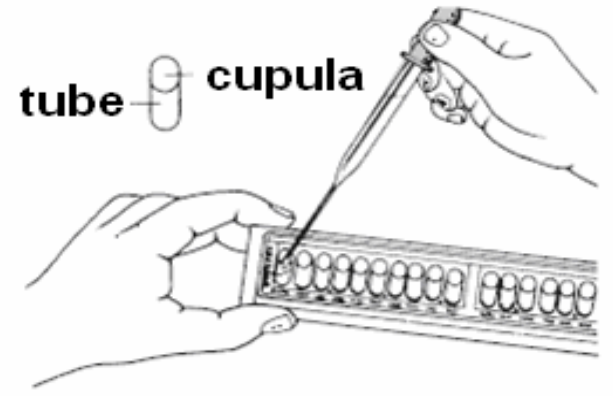


Figure 33 — Miniaturized systems

Each method consists of a plastic tube or strip that contains many different media to be inoculated and incubated (see figure 33). To facilitate rapid identification, these systems utilize numerical coding systems that can be applied to charts or computer programs. As convenient as these systems are, one must not assume that the conventional macromethods are becoming obsolete. Macromethods must still be used for culture studies and confirmatory tests; confirmatory tests by macromethods are often necessary when a particular test on a miniaturized system is in question. Another point to keep in mind is that all of the miniaturized multitest systems have been developed for the identification of *medically important* microorganisms. If one is trying to identify a saprophytic organism of the soil, water, there is no substitute for the conventional methods.

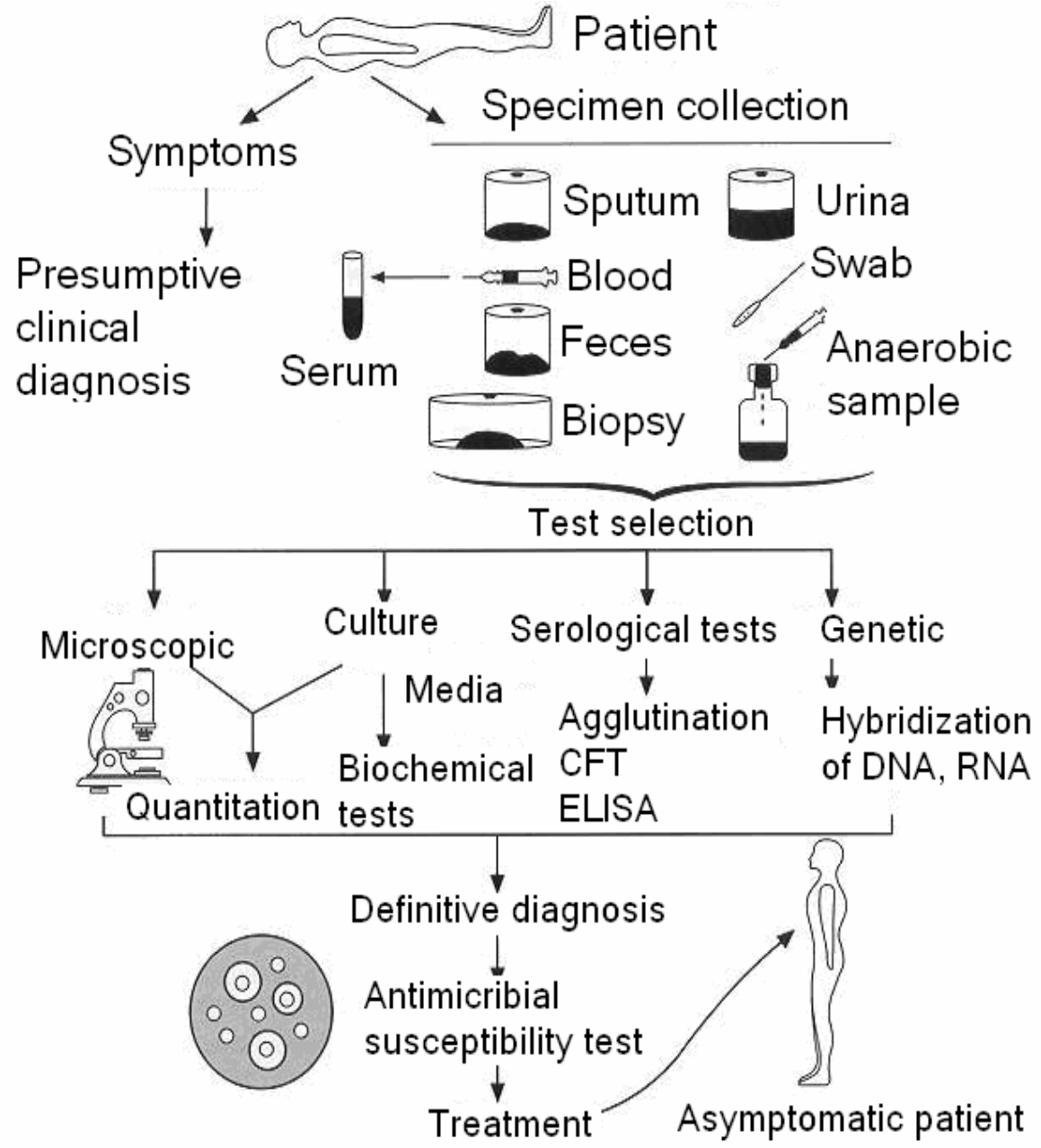


Figure 34 — Laboratory procedures used in confirming a clinical diagnosis of infectious disease with a bacterial etiology

Conclusion

It is necessary to use microbiologic laboratory methods to identify a specific etiologic agent. Diagnostic medical microbiology is the discipline that identifies etiologic agents of disease. The job of the clinical microbiology laboratory is to test specimens from patients for microorganisms that are, or may be, a cause of the illness and to provide information (when appropriate) about the in vitro activity of antimicrobial drugs against the microorganisms identified (see figure 34).

The staff of a clinical microbiology laboratory should be qualified to advise the physician as well as process specimens. The physician should supply salient information about the patient, such as age and sex, tentative diagnosis or details of the clinical syndrome, date of onset, significant exposures, prior antibiotic therapy, immunologic status, and underlying conditions.

The clinical microbiologist participates in decisions regarding the microbiologic diagnostic studies to be performed, the type and timing of specimens to be collected, and the conditions for their transportation and storage.

Above all, the clinical microbiology laboratory, whenever appropriate, should provide an interpretation of laboratory results.

The control of microbial growth

Microorganisms are ubiquitous. Since they cause contamination, infection and decay, it becomes necessary to remove or destroy them from material or from areas. **"Control of growth"** means to prevent growth of microorganisms. This control is effected in two basic ways: (1) by *killing microorganisms* or (2) by *inhibiting the growth* of microorganisms. Control of growth usually involves the use of physical or chemical agents which either kill or prevent the growth of microorganisms. Agents which kill cells are called **cidal** agents; agents which inhibit the growth of cells (without killing them) are referred to as **static** agents. Thus the term **bactericidal** refers to killing bacteria and **bacteriostatic** refers to inhibiting the growth of bacterial cells. A **bactericide** kills bacteria; a **fungicide** kills fungi and so on.

Sterilization is the complete destruction or elimination of all living microorganisms (*vegetative* and *spore* state!) from an object, surface or medium. This process is used in microbiology for preventing contamination by extraneous organisms, in surgery for maintaining asepsis, in food and drug manufacture for ensuring safety from contaminating agents and in many other situations. There are no degrees of sterilization: an object is either sterile or not. Sterility as a *result of sterilization* is absence of vegetative and spore forms if microorganisms on the environmental objects, surfaces and mediums.

Disinfection means the destruction or removal of all pathogenic microorganisms or organisms capable to give a rise of infection (often only vegetative forms!). The term **antisepsis** is used to indicate the prevention of infection usually by inhibiting the growth of bacteria in *wounds* and *tissues*. *Chemical disinfectants* which can be *safely* applied to *skin or mucous membrane* and are used for prevent infection by inhibiting the growth of bacteria are called **antiseptics**. **Decontamination** refers to the process of clearing an object or area from contaminants (microbes, chemical, radioactive and other dangers).

Methods of Sterilization (control of growth by physical agents)

Heat: Most important and widely used. For sterilization always consider *type of heat*, *time of application* and *temperature to ensure destruction of all microorganisms ("**thermal death time**")*. The sterilization time is related to the number of microorganisms in the suspension, presence or absence of spores and characteristics of given microorganism. Endospores of bacteria are considered the most thermoresistant of all cells so their destruction guarantees sterility.

The killing effect of heat is due to protein denaturation, coagulation and oxidative damage of the cell. In the case of spore, steam condenses on it, increasing its water content with ultimate hydrolysis and breakdown of the bacterial protein. **Dry heat** includes flaming, incineration and hot air method. **Moist heat** includes pasteurization, boiling and steam under the pressure method.

I**ncineration**: Burns organisms and physically destroys them. Used for needles, inoculating wires, glassware, etc. and objects not destroyed in the incineration process.

Flaming: Inoculating loop, the tip of forceps and searing spatulas are held in a Bunsen flame till they become red hot.

Boiling: 100^{o} for 30 minutes. Kills everything except some endospores (actually, for the purposes of purifying drinking water 100^{o} for five minutes is probably adequate though there have been some reports that Giardia cysts can survive this process). To kill endospores, and therefore sterilize the solution, very long or intermittent boiling is required.

Autoclaving (steam under pressure or pressure cooker): 121^{o}C for 15 minutes. Good for sterilizing almost anything (dressing, instruments, laboratory ware), but heat-labile substances will be denatured or destroyed. The principle of the autoclave is that water boils when its vapour pressure equals that of surrounding atmosphere. Hence when pressure inside a closed vessel increases, the temperature at which water boils also increases. Steam has penetrative power, condenses on a cooler surface and gives up its latent heat to that surface. The condenser water ensures moist conditions for killing microbes.

Radiation: usually destroys nucleic acids. Ultraviolet light is usually used (commonly used to sterilize the surfaces of objects), although x-rays and microwaves are possibly useful. Direct sunlight has an active bactericidal effect due to the combined effect of ultraviolet and heat rays.

Low temperature (refrigeration and freezing): Most organisms grow very little or not at all at 0o. Store perishable foods at low temperatures to slow rate of growth and consequent spoilage (e.g. milk). Low temperatures are not bactericidal. Psychrotrophs, rather than true psychrophiles, are the usual cause of food spoilage in refrigerated foods.

Table 6 — Methods of sterilization

Treatment	Temperature	Effectiveness
Incineration	>500º	Vaporizes organic material on nonflammable surfaces but may destroy many substances in the process
Boiling	100º	30 minutes of boiling kills microbial pathogens and vegetative forms of bacteria but may not kill bacterial endospores
Intermittent boiling	100º	Three 30-minute intervals of boiling, followed by periods of cooling kills bacterial endospores
Autoclave and pressure cooker (steam under pressure)	121º/15 minutes	Kills all forms of life including bacterial endospores
Dry heat (hot air oven)	160º/2 hours	For materials that must remain dry and which are not destroyed at T between 120º and 170º Good for glassware, metal, not plastic or rubber items
Pasteurization (batch method)	63º/30 minutes	Kills most vegetative bacterial cells including pathogens such as streptococci, staphylococci and Mycobacterium tuberculosis
Pasteurization (flash method)	72º/15 seconds	Effect on bacterial cells similar to batch method; for milk, this method is more conducive to industry

Drying (removal of H_2O): Most microorganisms cannot grow at reduced water activity. Often used to preserve foods (e.g. fruits, grains, etc.). Methods involve removal of water from product by heat, evaporation, freeze-drying, addition of salt or sugar. Spores are unaffected by drying.

Filtration: involves the physical removal of all bacteria from heat labile liquid, especially important to sterilize solutions which would be denatured by heat (e.g. antibiotics, injectable drugs, amino acids, vitamins, etc.). As viruses pass through ordinary filters, filtration can be used to obtain bacteria-free filtrates of clinical samples for virus isolation. Bacterial toxins can be obtained by passing cultures through filters.

Chemical and gas: (formaldehyde, glutaraldehyde, ethylene oxide) toxic chemicals kill all forms of life in a specialized gas chamber.

Control of microbial growth by chemical agents

Antimicrobial agents are chemicals that kill or inhibit the growth microorganisms. Antimicrobial agents include chemical preservatives and antiseptics, as well as drugs used in the treatment of infectious diseases of plants and animals. Antimicrobial agents may be of natural or synthetic origin, and they may have a static or cidal effect on microorganisms.

Types of antimicrobial agents

1. **Antiseptics**: microbicidal agents harmless enough to be applied to the skin and mucous membrane; should not be taken inside. Examples: mercurials, silver nitrate, iodine solution, alcohols, detergents.

2. **Disinfectants**: Agents that kill microorganisms, but not necessarily their spores, not safe for application to living tissues; they are used on inanimate objects such as tables, floors, utensils, etc. Examples: chlorine, hypochlorites, chlorine compounds, lye, copper sulfate, quaternary ammonium compounds. Disinfectants must be effective, accessible and safe.

Table 7 — Common antiseptics and disinfectants

Chemical	Action	Uses
Ethanol (50–70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Formaldehyde (8%)	Reacts with NH_2, SH and COOH groups	Disinfectant, kills endospores
Tincture of Iodine (2% I_2 in 70% alcohol)	Inactivates proteins	Antiseptic used on skin
Chlorine (Cl_2) **gas**	Forms hypochlorous acid (HClO), a strong oxidizing agent	Disinfect drinking water; general disinfectant
Silver nitrate ($AgNO_3$)	Precipitates proteins	General antiseptic and used in the eyes of newborns
Detergents (e.g. quaternary ammonium compounds)	Disrupts cell membranes	Skin antiseptics and disinfectants
Phenolic compounds (e.g. carbolic acid)	Denature proteins and disrupt cell membranes	Antiseptics at low concentrations; disinfectants at high concentrations

3. **Preservatives**: *static agents used to inhibit the growth of microorganisms, most often in foods*. If eaten they should be nontoxic. Examples: calcium propionate, sodium benzoate, formaldehyde, nitrate, sulfur dioxide.

4. **Chemotherapeutic agents**: *antimicrobial agents of synthetic origin useful in the treatment of microbial or viral disease*. Examples: sulfonilamides, isoniazid, ethambutol, chloramphenicol. Note that the microbiologist's definition of a chemotherapeutic agent requires that the agent be used for antimicrobial purposes and so excludes synthetic agents used for therapy against diseases that are not of microbial origin.

5. **Antibiotics**: *antimicrobial agents produced by microorganisms that kill or inhibit other microorganisms*. This is the microbiologist's definition. A more broadened definition of an antibiotic includes any chemical of natural origin (from any type of cell) which has the effect to kill or inhibit the growth of other types cells. Since most clinically-useful antibiotics are produced by microorganisms and are used to kill or inhibit infectious bacteria, we will follow the classic definition.

Control of microbial growth by biological factors

Microbial antagonism, in which one organism is inhibited and the other is unaffected. Usually, the inhibitor produces a substance that inhibits or kills one or more organisms. The substance may be specific in its action, affecting only a few species, or it may be nonspecific, affecting a large number of organisms.

Two or more organisms acting together to produce a substance that none can produce separately is a **synergistic relationship**. Such relationships are not uncommon among microorganisms. This phenomenon is readily demonstrated in the ability of some bacteria acting, synergistically, to produce gas by fermenting certain disaccharides.

There are many **commensalistic relationships** that exist between organisms in a mixed microbial population. The excretory products of one organism often become the nutrients of another. The oxygen usage of one species may produce the desired oxidation-reduction potential for another organism. In all cases of commensalism, the beneficiary contributes nothing in the way of benefit or injury to the other.

Antimicrobial agents

Most microbiologists distinguish two groups of antimicrobial agents used in the treatment of infectious disease: **antibiotics**, which are natural substances produced by certain groups of microorganisms, and **chemotherapeutic agents**, which are chemically synthesized. A hybrid substance is a **semisynthetic antibiotic**, wherein a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties.

Furthermore, some antimicrobial compounds, originally discovered as products of microorganisms, can be synthesized entirely by chemical means. They might be referred to as **synthetic antibiotics** to distinguish them from the chemotherapeutic agents.

The most important property of an antimicrobial agent, from a host point of view, is its **selective toxicity**, i.e., that the agent acts in some way that inhibits or kills bacterial pathogens but has little or no toxic effect on the host. This implies that the biochemical processes in the bacteria are in some way different from those in the animal cells, and that the advantage of this difference can be taken in chemotherapy.

Antibiotics are low-molecular weight substances that are produced as secondary metabolites by certain groups of microorganisms, especially *Streptomyces*, *Bacillus*, and a few molds (*Penicillium* and *Cephalosporium*) that are inhabitants of soils.

Clinically-useful antibiotic should have as many of these characteristics as possible:

1. It should have a wide spectrum of activity with the ability to destroy or inhibit many different species of pathogenic organisms.
2. It should be nontoxic to the host and without undesirable side effects.
3. It should be nonallergenic to the host.
4. It should not eliminate the normal flora of the host.
5. It should be able to reach the part of the human body where the infection is occurring.
6. It should be inexpensive and easy to produce.
7. It should be chemically-stable (have a long shelf-life).
8. Microbial resistance is uncommon and unlikely to develop.

Classification of antibiotics according to groups of sensitive microorganisms:

1. Antibacterial.
2. Antiviral.
3. Antifungal (pyrimidines, imidazole).
4. Antitumoral.
5. Antiprotozoal (metronidazole or trichopol).

Antimicrobial effects on cells

Antibiotics may have a **cidal (killing) effect** or a **static (inhibitory) effect** on a range of microbes. The decision to use a bactericidal or bacteriostatic drug to treat infection depends entirely upon the type of infection. For example, bactericidal drugs will only kill cells that are actively growing. Bacteriostatic drugs, in comparison, will only inhibit the growth of cells; ultimate elimination of the organisms is dependent upon host phagocytic activity. Some examples of bactericidal and bacteriostatic drugs are listed below.

Table 8 — Action of antibiotics

Bactericidal Drugs	Bacteriostatic Drugs
Streptomycin	Sulfonamides
Aminoglycosides	Tetracycline
Penicillin	Chloramphenicol

Antimicrobials can also be classified by their range of activity (**spectrum of action**). In general, five classifications can be described. The first of these is termed *narrow spectrum*. Narrow spectrum drugs are only active against a relatively small number of organisms. In general, narrow spectrum antibiotics are effective against Gram-positive organisms. The second classification is termed *moderate spectrum*. These drugs are generally effective against the Gram-positives and most systemic, enteric and urinary tract Gram-negative pathogens. The beta-lactam antibiotics (penicillin, ampicillin, cephalosporins, etc.) belong in a third classification, *narrow and moderate spectrum* because some members are only effective against Gram-positive organisms while other members can also kill certain Gram-negative bacteria. A fourth classification is termed *broad spectrum*. These drugs are effective against all prokaryotes with two

exceptions: *Mycobacteria* (see below) and *Pseudomonas*. The fifth group includes those drugs that are effective against *Mycobacteria*. The following table details some examples of these antimicrobials. If effective against a single organism or disease, they are referred to as **limited spectrum**.

The following discussion of antibiotics and chemotherapeutic organizes the **antimicrobial agents based on their mode of action in bacterial cells** (*site of antibiotic activity within the target cell*). The various cellular targets include the cell wall, the plasma membrane, the nucleic acids and proteins.

Table 9 — Range of activity of different antibiotics

Range of activity	Organisms affected	Example antibiotics
Narrow spectrum	Gram-positives (*Actinomyces*, *Corynebacteria*, *Bacillus*, *Clostridium)*, Pyogenic cocci, Spirochetes	Macrolides (Erythromycin) Polypeptides (Polymyxin)
Moderate spectrum	Gram-positives plus systemic, enteric and urinary tract Gram-negatives	Sulfonamides Aminoglycosides (Streptomycin, Gentamycin)
Narrow/moderate spectrum	Gram-positives plus Gram-negatives	Beta-lactams (Penicillin, Ampicillin, Cephalosporins)
Broad spectrum	All prokaryotes except *Mycobacteria* and *Pseudomonas*	Chloramphenicol Tetracycline
Anti-mycobacterial	*Mycobacteria*	Isoniazid Ethambutol Streptomycin

Cell wall synthesis inhibitors

Cell wall synthesis inhibitors generally inhibit some step in the synthesis of bacterial peptidoglycan. The beta lactam antibiotics are stereochemically related to D-alanyl-D-alanine which is a substrate for the last step in peptidoglycan synthesis, the final cross-linking between peptide side chains. Generally they exert their selective toxicity against eubacteria because human cells lack cell walls. Beta lactam antibiotics are normally *bactericida*l and require that cells be actively growing in order to show their toxicity.

Beta lactam antibiotics are the products of two groups of fungi, *Penicillium* and *Cephalosporium* molds, and are correspondingly represented by the **penicillins** and **cephalosporins**.

1. Penicillins bin and inhibit the carboxypeptidase and transpeptidase enzymes that are required for peptidoglycan biosynthesis.

Although nontoxic, penicillins occasionally cause death when administered to persons who are allergic to them. In the U.S. there are 300–500 deaths annually due to penicillin allergy. In allergic individuals the beta lactam molecule attaches to a serum protein which initiates an *IgE-mediated inflammatory response*.

Resistance to penicillins may be determined by the organism's production of penicillin-destroying enzymes (**beta-lactamases**). These enzymes have been described for many Gram positive and Gram negative bacteria. Some beta-lactamases are plasmid-mediated, while others are chromosomally mediated.

There are several classes of penicillins

▪ **Natural penicillins** are highly effective against Gram positive bacteria (and gonococcus), but are inactivated by the bacterial enzyme penicillinase. Examples include **penicillin G, F, X, K, O, and V**. They are considered *narrow spectrum* since they are not effective against Gram-negative rods.

▪ **Semisynthetic penicillins** are effective against *Gram positive* bacteria but are not inactivated by penicillinase. Examples include **methicillin, dicloxacillin**.

▪ **Semisynthetic broad-spectrum penicillins** are effective against a variety of *Gram positive* and *Gram negative* bacteria but are inactivated by penicillinase. Examples include **ampicillin, oxacillin**, **azlocillin, mezlocillin, and piperacillin**.

▪ **Semisynthetic broad-spectrum penicillins combined with beta lactamase inhibitors such as clavulanic acid and sulbactam**. Although the clavulanic acid and sulbactam have no antimicrobial action of their own, they inhibits penicillinase thus protecting the penicillin from degradation. Examples include **amoxicillin plus clavulanic acid and ampicillin plus sulbactam**.

2. Cephalolsporins are beta lactam antibiotics with a similar mode of action to penicillins that are produced by species of *Cephalosporium*. The have a low toxicity and a somewhat broader spectrum than natural penicillins. Cephalosporins are effective against a variety of *Gram positive* and *Gram negative* bacteria and are resistant to penicillinase (although some can be inactivated by other beta-lactamase enzymes similar to penicillinase). Three "generations" of cephalosporins have been developed over the years in an attempt to counter bacterial resistance.

First generation cephalosporins include **cephalothin, cephapirin and cephalexin**.

Second generation cephalosporins include **cefamandole, cefuroxime, and cefoxitin**.

Third generation cephalosporins include **cefotaxime, cefixime, ceftazidine, and moxalactam**.

3. Carbapenems: Carbapenems consist of a *broad spectrum* beta lactam antibiotic to inhibit peptidoglycan synthesis combined with cilastatin sodium, an agent which prevents degradation of the antibiotic in the kidneys. An example is **imipenem**.

4. Monobactams: Monobactams are *broad spectrum* beta lactam antibiotics resistant to beta lactamase. An example is **aztreonam**.

The latter are particularly useful for the treatment of allergic individuals. A person who becomes allergic to penicillin usually becomes allergic to the cephalosporins and the carbapenems as well. Such individuals can still be treated with the monobactams, which are structurally different so as not to induce allergy.

5. Bacitracin is a polypeptide antibiotic produced by *Bacillus* species. Bacitracin is used topically against *Gram positive* bacteria. It prevents cell wall growth by inhibiting the release of the mucopeptide subunits of peptidoglycan from the lipid carrier molecule that carries the subunit to the outside of the membrane. Teichoic acid synthesis, which requires the same carrier, is also inhibited. Bacitracin has a high toxicity which precludes its systemic use. It is present in many topical antibiotic preparations, and since it is not absorbed by the gut, it is given to "sterilize" the bowel prior to surgery.

6. Cycloserine inhibits the early stages of murein synthesis. Cycloserine enters bacterial cells by means of an active transport system for glycine and can reach a relatively high intracellular concentration. This concentrating effect, along with its high affinity for susceptible enzymes, enables cycloserine to function as a very effective antimicrobial agent. However, it is fairly toxic and has limited use as a secondary drug for tuberculosis.

7. Glycopeptides (produced by the bacterium *Streptomyces*): **Vancomycin** and **teichoplanin** are glycopeptides that are effective against *Gram positive* bacteria.

However, it has become important in clinical usage for treatment of infections by strains of *Staphylococcus aureus* that are resistant to virtually all other antibiotics.

Cell membrane inhibitors

These antibiotics disorganize the structure or inhibit the function of bacterial membranes. The integrity of the cytoplasmic and outer membranes is vital to bacteria, and compounds that disorganize the membranes rapidly kill the cells. However, due to the similarities in phospholipids in eubacterial and eukaryotic membranes, this action is rarely specific enough to permit these compounds to be used systemically. The only antibacterial antibiotic of clinical importance that acts by this mechanism is **polymyxin**, produced by *Bacillus polymyxis*. Polymyxin is effective mainly against *Gram-negative* bacteria. The balance between effectiveness and damage to the kidney and other organs is dangerously close, and the drug should only be given under close supervision in the hospital.

Nystatin (produced by the bacterium *Streptomyces*): Nystatin is used mainly for *Candida* yeast infections. **Imidazoles** (produced by the bacterium *Streptomyces*): The imidazoles are antifungal antibiotics used for yeast infections, dermatophytic infections, and systemic fungal infections. **Amphotericin B** (produced by the bacterium *Streptomyces*): Amphotericin B is used for systemic fungal infections.

Protein synthesis inhibitors

Many therapeutically useful antibiotics show their action to inhibition of some step in the complex process of protein synthesis. Their attack is always at one of the events occurring on the ribosome and never at the stage of amino acid activation or attachment to a particular tRNA. Most have an affinity or specificity for 70S (as opposed to 80S) ribosomes, and they achieve their selective toxicity in this manner. The most important antibiotics with this mode of action are the **tetracyclines, chloramphenicol**, the **macrolides** (e.g. erythromycin) and the **aminoglycosides**.

1. **Aminoglycosides** are products of *Streptomyces species* and are represented by **streptomycin** and **gentamicin**. These antibiotics exert their activity by binding to bacterial ribosomes and preventing the initiation of protein synthesis. Aminoglycosides have been used against a wide variety of bacterial infections caused by *Gram-positive* and *Gram-negative* bacteria. Streptomycin has been used extensively as a primary drug in the treatment of tuberculosis. **Gentamicin** is active against many strains of *Gram-positive* and *Gram-negative* bacteria, including some strains of *Pseudomonas aeruginosa.*

2. **Tetracyclines** are all natural products of *Streptomyces,* although some can now be produced semisynthetically or synthetically. **Tetracycline** and **doxycycline** are the best known. The tetracyclines are broad-spectrum antibiotics with a wide range of activity against both *Gram-positive* and *Gram-negative bacteria*. Tetracyclines inhibit protein synthesis on isolated 70S or 80S (eukaryotic) ribosomes. However, most bacteria possess an active transport system for tetracycline that will allow intracellular accumulation of the antibiotic at concentrations 50 times as great as that in the medium. This greatly enhances its antibacterial effectiveness and accounts for its specificity of action, since an effective concentration cannot be accumulated in animal cells. Thus a blood level of tetracycline which is harmless to animal tissues can halt protein synthesis in invading bacteria.

The tetracyclines have a low toxicity and minimal side effects when taken by animals. The combination of their broad spectrum and low toxicity has led to their overuse and misuse by the medical community and the wide-spread development of resistance

has reduced their effectiveness. Nonetheless, tetracyclines still have some important uses, such as the use of **doxycycline** in the treatment of Lyme disease.

3. Chloramphenicol is a protein synthesis inhibitor has a broad spectrum of activity but it exerts a bacteriostatic effect. It is effective against intracellular parasites such as the *rickettsiae*. Chloramphenicol was originally discovered and purified from the fermentation of a *Streptomyces*, but currently it is produced entirely by chemical synthesis. Chloramphenicol inhibits the bacterial enzyme, thereby preventing the growth of the polypeptide chain during protein synthesis.

The eukaryotic cells may be inhibited by chloramphenicol are those undergoing rapid multiplication, thereby rapidly synthesizing mitochondria (unfortunate toxicity). Such cells include the blood forming cells of the bone marrow, the inhibition of which could present as aplastic anemia. Now it is seldom used in human medicine except in life-threatening situations (e.g. typhoid fever).

4. Macrolide family. The most important members of the group are **erythromycin** is active against most *Gram-positive bacteria*, *Neisseria, Legionella and Haemophilus*, but not against the Enterobacteriaceae. Macrolides are bacteriostatic for most bacteria.

Effects on nucleic acids

Some antibiotics and chemotherapeutic agents affect the synthesis of DNA or RNA, or can bind to DNA or RNA so that their messages cannot be read. Either case, of course, can block the growth of cells. Two nucleic acid synthesis inhibitors which have selective activity against procaryotes and some medical utility are the **quinolones** and **rifamycins**.

1. **Nalidixic acid** is a *synthetic chemotherapeutic agent* which has activity mainly against *Gram-negative* bacteria. Nalidixic acid belongs to a group of compounds called **quinolones.** Some quinolones penetrate macrophages and neutrophils better than most antibiotics and are thus useful in treatment of infections caused by intracellular parasites. However, the main use of nalidixic acid is in treatment of lower urinary tract infections.

Some quinolones have a broadened spectrum against *Gram-positive* bacteria. The **fluoroquinolone** (ciprofloxacin) was recently touted as the drug of choice for treatment and prophylaxis of anthrax, which is caused by a Gram-positive bacillus.

2. Rifamycins are a comparatively new group of antibiotics, also the products of *Streptomyces*. **Rifampicin** is a semisynthetic derivative of **rifamycin** that is active against *Gram-positive* bacteria (including Mycobacterium tuberculosis) and some *Gram-negative* bacteria. **Rifampicin** acts quite specifically on the bacterial RNA polymerase and is inactive towards DNA polymerase or RNA polymerase from animal cells. It has been found to have greater bactericidal effect against *M .tuberculosis* than other anti-tuberculosis drugs, and it has largely replaced **isoniazid** as one of the front-line drugs used to treat the disease, especially when isoniazid resistance is indicated. It is effective orally and penetrates the cerebrospinal fluid so it is useful for treatment of bacterial meningitis.

Competitive inhibitors

Many of the synthetic chemotherapeutic agents are **competitive inhibitors** of *essential metabolites* or *growth factors* that are needed in bacterial metabolism. Hence, these types of antimicrobial agents are sometimes referred to as **antimetabolites** or **growth factor analogs**, since they are designed to specifically inhibit an essential metabolic pathway in the bacterial pathogen. At a chemical level, competitive inhibitors are structurally similar to a bacterial growth factor or metabo-

lite, but they do not fulfill their metabolic function in the cell. Some are bacteriostatic and some are bactericidal. Their selective toxicity is based on the premise that the bacterial pathway does not occur in the host.

Sulfonamides were introduced as *chemotherapeutic agents* by Domagk in 1935, which showed that one of these compounds had the effect of curing mice with infections caused by beta-hemolytic streptococci. Chemical modifications of the compound **sulfanilamide** gave compounds with even higher and broader antibacterial activity. Bacteria which are almost always sensitive to the sulfonamides include *Streptococcus pneumoniae*, beta-hemolytic streptococci and *E. coli*. The sulfonamides have been extremely useful in the treatment of meningococcal meningitis (because they cross the blood-brain barrier).

The sulfonamides (e.g. **Trimethoprim)** are inhibitors of the bacterial enzymes required for the synthesis of tetrahydofolic acid (THF), the vitamin form of folic acid essential for 1-carbon transfer reactions. Sulfonamides are structurally similar to paraaminobenzoic acid (PABA), the substrate for the first enzyme in the THF pathway, and they competitively inhibit that step. Trimethoprim is structurally similar to dihydrofolate (DHF) and competitively inhibits the second step in THF synthesis mediated by the DHF reductase. Animal cells do not synthesize their own folic acid but obtain it in a preformed fashion as a vitamin. Since animals do not make folic acid, they are not affected by these drugs, which achieve their selective toxicity for bacteria on this basis.

Microorganisms that produce antibiotics

The molds, ***Penicillium*** and ***Cephalosporium,*** produce Beta-lactam antibiotics, i.e., penicillin, cephalosporin, and their relatives. Actinomycetes, mainly ***Streptomyces*** species: produce tetracyclines, aminoglycosides (streptomycin and its relatives), macrolides (erythromycin and its relatives), chloramphenicol, rifamycins, and most other clinically-useful antibiotics that are not beta-lactams. **Bacillus** species, such as *B. polymyxa* and *Bacillus subtilis* produce polypeptide antibiotics (e.g. polymyxin and bacitracin). These organisms all have in common that they live in a soil habitat and they form some sort of a spore or resting structure. It is not known why these microorganisms produce antibiotics. It may rest in the obvious, i.e., the antibiotics afford the microbes some nutritional advantage in their habitat by antagonism against the competition. However, it may rest on the subtle: i.e., the antibiotics act as some sort of hormone or signal molecule associated with sporulation or dormancy or germination.

Classification of antibiotics according to source of obtaining

1. Fugal origin (**penicillins** produced by Penicillium and **cephalosporins** produced by Cephalosporium).
2. Actinomycetes origin (Streptomyces produce 80% of all **natural antibiotics**).
3. Bacterial origin (Bacillus, Pseudomonas produce **gramicidin, polymyxin**).
4. Animal origin (lysozyme, interferon).
5. Plant origin (phytoncides)
6. Synthetic origin (quinolones and fluoroquinolones).

Antibiotics are *secondary metabolites* of microorganisms and they are produced at the same time that the cells begin sporulation processes. Antibiotics tend to be rather large, complicated, organic molecules and may require as many as 30

separate enzymatic steps to synthesize. The maintenance of a substantial component of the bacterial genome devoted solely to the synthesis of an antibiotic leads one to the conclusion that the process is important, if not essential, to the survival of these organisms in their natural habitat.

Most of the microorganisms that produce antibiotics are resistant to the action of their own antibiotic, although the organisms are affected by other antibiotics and their antibiotic may be effective against closely-related strains. **Bacteriocins** are microbial antibiotics which inhibit the growth of closely-related strains. Ability of bacteria to produce the bacteriocins (**bacteriocinogenicity**) is in the base of bacterial **antagonism**.

The medical problem of bacterial drug resistance

Obviously, if a bacterial pathogen is able to develop or acquire resistance to an antibiotic, then that substance becomes useless in the treatment of infectious disease caused by that pathogen (unless the resistance can somehow be overcome with secondary measures). So as pathogens develop resistance, we must find new (different) antibiotics to fill the place of the old ones in treatment regimes. Hence, natural penicillins have become useless against staphylococci and must be replaced by other antibiotics; tetracycline, having been so widely used and misused for decades, has become worthless for many of the infections that once designated it as a "wonder drug".

Not only is there a problem in finding new antibiotics to fight old diseases (because resistant strains of bacteria have emerged), there is a parallel problem to find new antibiotics to fight new diseases. In the past two decades, many "new" bacterial diseases have been discovered (Legionnaire's disease, gastric ulcers, Lyme disease, toxic shock syndrome, "skin-eating" streptococci). We are only now able to examine patterns of susceptibility and resistance to antibiotics among new pathogens that cause these diseases. Broad patterns of resistance exist in these pathogens, and it seems likely that we will soon need new antibiotics to replace the handful that are effective now against these bacteria, especially as resistance begins to emerge among them in the selective environment antibiotic chemotherapy.

There are many different mechanisms by which microorganisms can exhibit resistance to drug:

1. Microorganisms produce enzymes that destroy the active drug. E.g. Staphylococci resistant to natural penicillin produce a beta-lactamase.
2. Microorganisms change their permeability to the drug. E.g. Tetracyclines accumulate in sensitive bacteria but not in resistant bacteria.
3. Microorganisms develop an altered (changeable) structural target for the drug. E.g. resistance to penicillin may be function of the loss of penicillin-binding proteins in the bacterial cell wall.
4. Microorganisms develop altered metabolic pathways that bypass the reaction inhibited by the drug. E. g. Some sulfonamide-resistant bacteria do not require paraaminobenzoic acid (PABA) but, like mammalian cells, can use folic acid.
5. Microorganisms develop altered enzymes that can still perform its metabolic function but is much less affected by the drug.

Nongenetic origin of drug resistance:

1. Active replication of bacteria is required for most antibacterial drug action. Consequently, microorganisms that are not active (nonmultiplying) *may be phenotypically resistant to drugs*. E. g. “persisting” Mycobacteria for many years after infec-

tion and do not multiply are resistant to treatment, but if they start to multiply (suppression of immunity) they are sensitive to some drugs.

2. Microorganisms *may lose the specific target structure* for a drug for several generations. Penicillin-sensitive bacteria lacking cell wall (formation of L-forms) become are resistant to cell wall inhibitors.

3. Microorganisms *may infect the host sites where antimicrobials are excluded or are not active*. Gentamicin is not effective in treating salmonella enteric fevers because the salmonellae are intracellular and gentamicin does not enter the cells.

Genetic origin of drug resistance:

1. *Chromosomal resistance* develops as a result of spontaneous mutation in a locus that control the sensitivity to a given drug. The presence of the drug serves as a selecting mechanism to suppress sensitive organisms and for the growth of drug-resistant mutants. Mutation in the gene controlling receptor-protein results in streptomycin resistance.

2. Extrachromosomal resistance. Bacteria are able to exchange genes in nature by three processes: conjugation, transduction and transformation. Genetic recombination can follow the transfer of DNA from one cell to another leading to the emergence of a new genotype (recombinant). It is common for DNA to be transferred as *plasmids* between mating bacteria. Since bacteria usually develop their genes for **multiple drug resistance** (for *several antibiotics*) on **R-plasmids** (called **resistance transfer factors** or **RTFs**), they are able to spread drug resistance to other strains and species during genetic exchange processes. Plasmid genes often control the formation of enzymes capable of destroying the antimicrobial drugs. Thus, plasmids determine resistance to penicillin by carrying genes or the formation of beta-lactamases. **Transposons** (genetic elements) provide the resistance to a *one antibiotic*.

Escherichia coli, Proteus, Salmonella, Shigella and *Pseudomonas* are examples of bacteria which frequently have R-plasmids. Because of the problem of antibiotic resistance, **antibiotic susceptibility testing** is usually done in the clinical laboratory to determine which antimicrobial chemotherapeutic agents will most likely be effective on a particular strain of microorganism

The combined effects of fast growth rates, high concentrations of cells, genetic processes of mutation and selection, and the ability to exchange genes, account for the extraordinary rates of adaptation and evolution that can be observed in the bacteria. For these reasons bacterial adaptation (resistance) to the antibiotic environment seems to take place very rapidly in evolutionary time.

Dangers from indiscriminate use of antimicrobial chemotherapeutic agents or by-effects of antibiotic therapy:

1. Drug toxicity (endotoxic shock);
2. If a choice is available, a narrow spectrum is preferable since it will cause less destruction to the body's normal flora. In fact, indiscriminate use of broad-spectrum antibiotics can lead to **superinfection** by **opportunistic microorganisms** (relative-pathogenic microorganisms), such as *Candida* (yeast infections) and *Clostridium difficile* (antibiotic-associated ulcerative colitis), when the body's normal flora is destroyed;
3. Allergic reactions to the drug;
4. Immunosuppressive reactions;
5. Mutagenic action on the fetus;
6. Appearance of the atipic forms of bacteria which are difficult to identify (e.g. L-forms);
7. Selection for resistant strains of microorganisms (antibiotic resistance).

Principles of rational antibiotic therapy

◦ **Microbiological principle:** to apply antibiotics only at presence of doctors advice and do not apply the antibiotics for preventive maintenances.

◦ **Pharmacological principle:**

1.To observe of dosage and manner of the injection of the drug (subtherapeutic doses cause the drug resistance);

2. To take into account the possibility of combination between drug and other medicinal preparations;

3.Duration of antibiotic therapy (change of a preparation in 10–15 days);

4.Knowledge of the ways of drug removing from the organism.

◦ **Clinical principl**e: to apply antibiotics taking into account the state of a patient.

◦ **Epidemiological principle**: to take into account the microbial resistance to a given antibiotic.

Antibiotic susceptibility testing

For some microorganisms, susceptibility to chemotherapeutic agents is predictable. However, for many microorganisms (*Pseudomonas, Staphylococcus aureus, Escherichia coli*, etc.) there is no reliable way of predicting which antimicrobial agent will be effective in a given case. This is especially true with the emergence of many antibiotic-resistant strains of bacteria. Because of this, antibiotic susceptibility testing is often essential in order to determine which antimicrobial agent to use against a specific strain of bacterium.

Several tests may be used to tell a physician which antimicrobial agent is most likely to combat a specific pathogen:

TUBE DILUTION TESTS: In this test, a series of culture tubes are prepared, each containing a liquid medium and a different concentration of a chemotherapeutic agent. The tubes are then inoculated with the test organism and incubated for 16–20 hours at 35C. After incubation, the tubes are examined for turbidity (growth). The lowest concentration of chemotherapeutic agent capable of preventing growth of the test organism is the **minimum inhibitory concentration (MIC)**.

Subculturing of tubes showing no turbidity into tubes containing medium but no chemotherapeutic agent can determine the **minimum bactericidal concentration (MBC)**. MBC is the lowest concentration of the chemotherapeutic agent that results in no growth (turbidity) of the subcultures. These tests, however, are rather time consuming and expensive to perform.

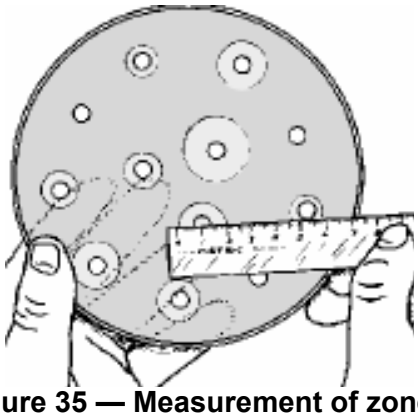


Figure 35 — Measurement of zones of growth inhibition

AGAR DIFFUSION TEST: A procedure commonly used in clinical labs to determine antimicrobial susceptibility is the **disc diffusion method**. In this test, the in vitro response of bacteria to a standardized antibiotic-containing disc has been correlated with the clinical response of patients given that drug. In the development of this method, a single high-potency disc of each chosen chemotherapeutic agent was used.

Zones of growth inhibition (see figure 35) surrounding each type of disc were correlated with the minimum inhibitory concentrations of each antimicrobial agent (as determined by the tube dilution test). The MIC for each agent was then compared to the usually-attained blood level in the patient with adequate dosage. Categories of "Resistant," "Intermediate," and "Sensitive" were then established.

The term **intermediate** generally means that the result is inconclusive for that drug-organism combination. The term **moderately susceptible** is usually applied to those situations where a drug may be used for infections in a particular body site, e.g., cystitis because the drug becomes highly concentrated in the urine.

Two methods for performing antibiotic susceptibility tests as illustrated in figure 36:

(A) **Disk diffusion method**. (B) **Minimum inhibitory concentration** (MIC) **method.**

In the example shown, two different microorganisms are tested by both methods against the same antibiotic. The MIC of the antibiotic for the susceptible microorganism is 8 μg/ml. The corresponding disk diffusion test shows a zone of inhibition surrounding the disk. In the second sample, a resistant microorganism is not inhibited by the highest antibiotic concentration tested (MIC > 16 μg/ml) and there is no zone of inhibition surrounding the disk. The diameter of the zone of inhibition is inversely related to the MIC.

Mechanisms to reduce antibiotic resistance:

1. Control, reduce or cycle antibiotic usage.
2. Improve hygiene in hospitals and among hospital personnel and reduce movement of patients to eliminate the dissemination of resistant organisms within hospitals.
3. Discover or develop new antibiotics.
4. Modify existing antibiotics chemically to produce compounds and study mechanisms of resistance.
5. Develop inhibitors of antibiotic-modifying enzymes.
6. Define agents that would “cure” resistance plasmids

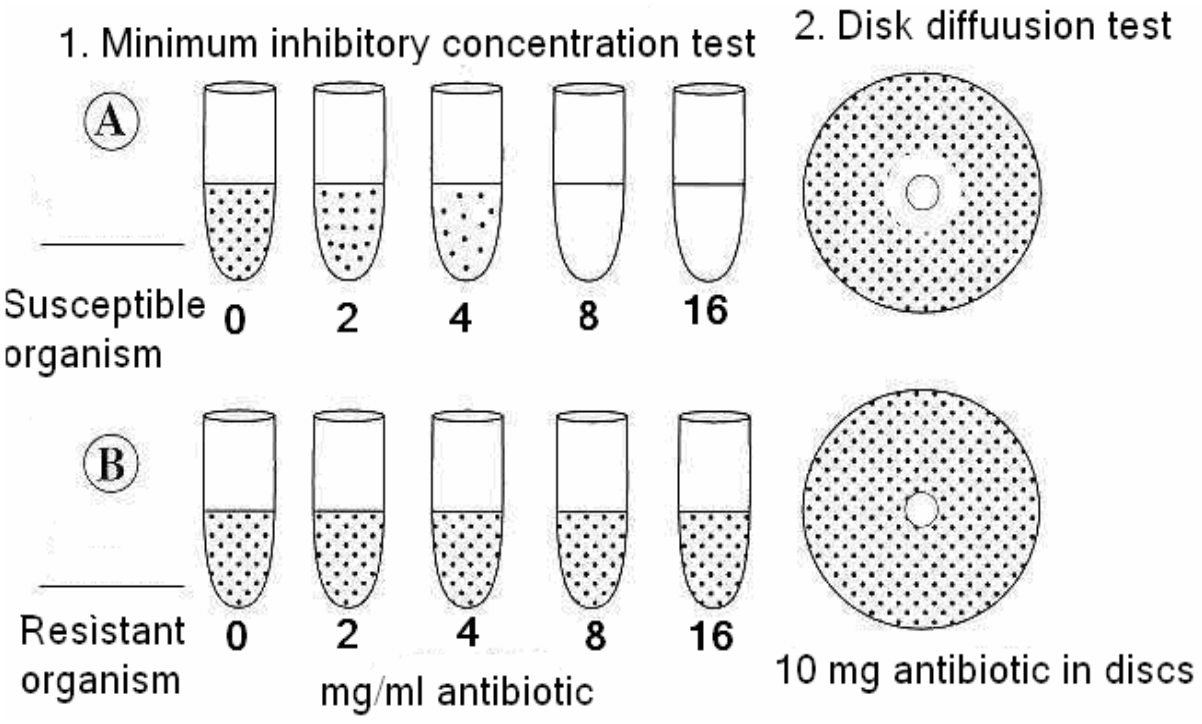
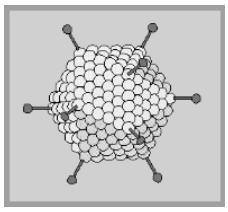


Figure 36 — Antibiotic susceptibility tests



GENERAL VIROLOGY

Unicellular microorganisms may be classified in descending order of complexity as *eukaryotes,* such as protozoa and fungi, and *prokaryotes,* such as bacteria, *mycoplasma, rickettsiae* and *chlamydiae*. Viruses do not fall strictly into the category of unicellular microorganisms as they do not possess a cellular organization. Even the simplest of microorganisms are cells enclosed within a cell wall, containing both types of nucleic acid (DNA and RNA), synthesising their own macromolecular constituents and multiplying by binary fission. Viruses, on the other hand, *do not have a cellular organization* and contain only *one type of nucleic acid*, either DNA or RNA but never both. They are *obligate intracellular parasites*. They lack the enzymes necessary for protein and nucleic acid synthesis and are dependent for replication on the synthetic machinery of host cells. They multiply by a *complex process* and not by binary fission. They are unaffected by antibacterial antibiotics. **In spite of these basic differences, viruses are generally considered microorganisms in medical microbiology.** As the smallest 'living units', viruses offer the best models for understanding the chemistry of “life”.

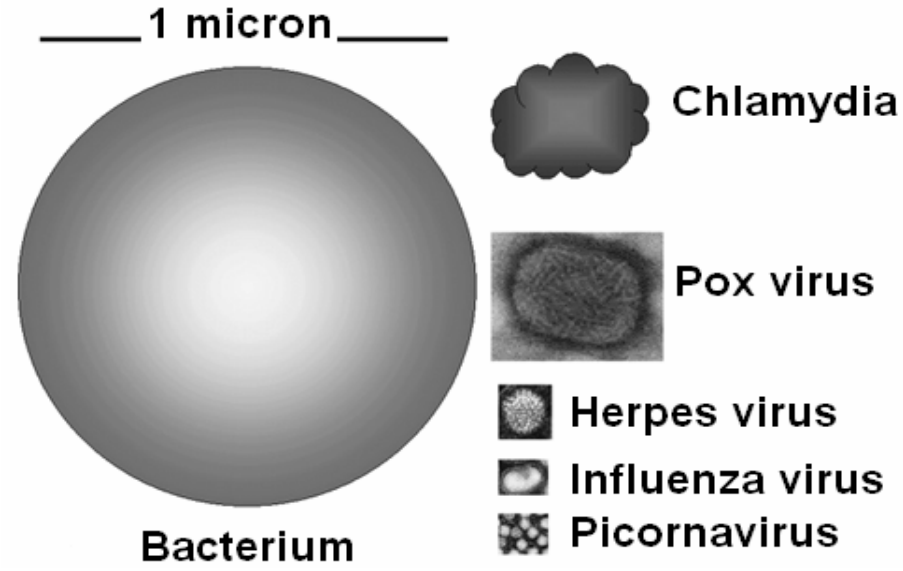


Figure 37 — Relative size of different microorganisms

The medical importance of viruses lies in their ability to cause a very large number of human diseases. Viral diseases range from minor diseases such as the common cold to terrifying diseases such as rabies or AIDS. They may be sporadic like mumps, endemic like infectious hepatitis, epidemic like dengue fever or pandemic like influenza.

The extracellular infectious virus particle is called the **virion**. Viruses are much smaller than bacteria (see figure 37). It was their small size and 'filterability' (ability to pass through filters that can hold back bacteria) that led to their recognition as a separate class of infectious agents. As they were too small to be seen under the light microscope, they were called 'ultramicroscopic'. Some of the larger viruses, such as

poxviruses, can be seen under the light microscope when suitably stained. The virus particles seen in this manner are known as **“elementary bodies”.**

Viruses vary widely in size. The largest among them (for example **poxviruses**) measuring about 300 nm, are as large as the smallest bacteria (mycoplasma). The smallest viruses (for example, **parvovirus**) measuring about 20 nm. The method of measuring virus size is **electron microscopy**. Purified preparations of virions may be examined under the electron microscope either unstained or stained.

There are also the **“unconventional agents”** sometimes knew as 'unconventional viruses' or 'atypical viruses' — the main kinds which have been studied so far are viroids and prions. **Viroids** are infectious agents that represent themselves a protein-free, low molecular weight **RNA** resistant to heat and organic solvents but sensitive to nucleases. It is possible that the causative agents of some animal and human diseases may turn out to belong to class of viroids. **Prions** are virus-like proteinaceous infectious agents, had been shown to be a small particles and probably 4–6 nm diameter, without any detectable nucleic acid, resistant to heat, UV rays and nucleases, and sensitive to proteases. They are may be responsible for some chronic neurological degenerative diseases of humans, scrapie Kuru and Cruetzfeidt-Jacob disease.

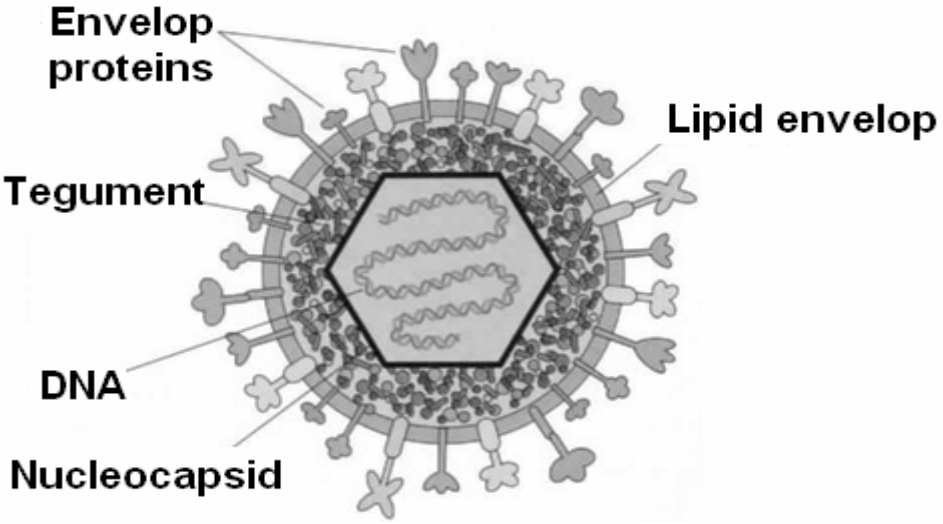


Figure 38 — Structure of viral particle

Structure and shape: Viruses consist of nucleic acid (DNA or RNA) surrounded by a protein coat called a **capsid**. The capsid is made up of individual structural subunits called **capsomeres**. The function of the capsid is to protect the nucleic acid from inactivation by nucleases and other deleterious agents in the environment. The combination of the nucleic acid genome enclosed in the capsid is called the **nucleocapsid** (see figure 38).

Two kinds of symmetry are encountered in the capsid — ***icosahedral*** (cubical) and ***helical****.* Two types of capsomers constitute the icosahedral capsid. They are the *pentagonal* capsomers at the vertices (*pentons*) and the *hexagonal* capsomers making up the facets (*hexons*). In the nucleocapsids with helical symmetry, the capsomers and nucleic acid are bound together to form a helical or spiral tube. The tube may be rigid, as in the tobacco mosaic virus but in the case of animal viruses, the tubular nucleocapsid is flexible. Not all viruses show the typical icosahedral or helical symmetry. Some, like the poxviruses, exhibit a **complex symmetry**.

Basic structural forms of viruses in nature

- *Naked icosahedral* e.g. poliovirus, adenovirus, hepatitis A virus;
- *Naked helical* e.g. tobacco mosaic virus, so far no human viruses with this structure known;
- *Enveloped icosahedral* e.g. herpes virus, yellow fever virus, rubella virus;
- *Enveloped helical* e.g. rabies virus, influenza virus, parainfluenza virus, mumps virus, measles virus;
- *Complex* e.g. poxvirus (see figure 39).

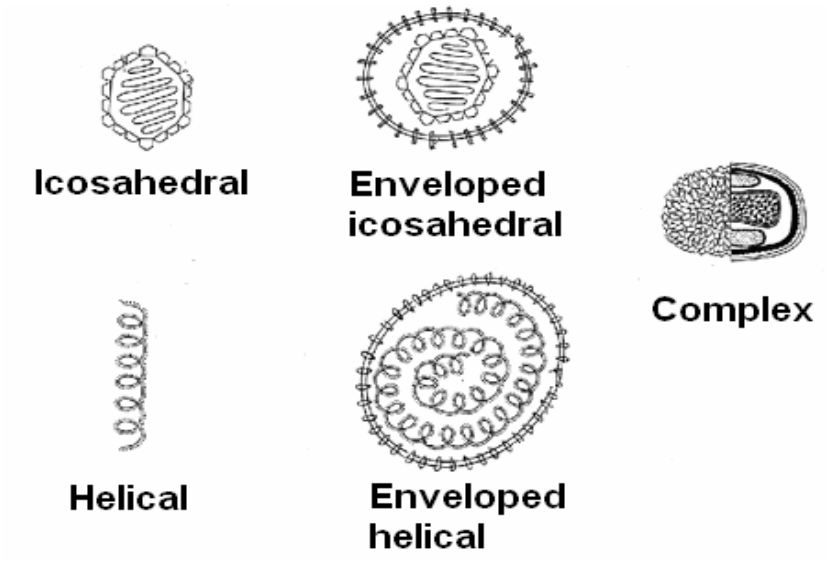


Figure 39 — Five basic types of viral symmetry

Virions may be enveloped virus (*complex* viruses) or nonenveloped (naked, *simple*). The **envelope** or outer covering of viruses is derived from the host cell membrane when the progeny virus is released by budding! The envelope is lipoprotein in nature. **Envelopes confer chemical, antigenic and biological properties on viruses**. Enveloped viruses are susceptible to the action of lipid solvents like chloroform and bile salts.

The overall shape of the virus particle varies in different groups of viruses. Most animal viruses are roughly spherical. Some are irregular and pleomorphic. The rabies virus is bullet shaped, Ebolovirus filamentous and poxviruses are brick shaped. The tobacco mosaic virus is rod shaped. Bacterial viruses have a complex morphology.

Chemical properties: Viruses contain only one type of nucleic acid, either single or double stranded DNA or RNA. In this respect, viruses are unique, for nowhere else in nature is genetic information exclusively carried by RNA. Viruses also contain protein which makes up the capsid. Viral protein, besides protecting the nucleic acid, also determines the antigenic specificity of the Virus. Enveloped viruses contain lipids derived from the host cell membrane. Some viruses also contain small amounts of carbohydrate. Most viruses do not possess any enzymes for the synthesis of viral components or for energy production but some have other enzymes, for example, the neuraminidase in the influenza virus. **Retrovirues** have a unique enzyme, RNA-dependent-DNA polymerase or '*transcriptase*' which can transcribe RNA into DNA.

Resistance: With few exceptions, viruses are very heat labile. There are individual variations but in general, they are inactivated within seconds at 56°C, minutes at 37°C and days at 4°C. They are stable at low temperatures! For long term

storage, they are kept frozen at –70°C. Some viruses (such as poliovirus) do not stand freeze drying. Viruses vary greatly in their resistance to acidity. For example, enteroviruses are very resistant to acid pH while rhinoviruses are very susceptible. All viruses are disrupted under alkaline conditions.

Viruses are inactivated by sunlight, UV rays and ionizing radiations. They are, in general, more resistant than bacteria to chemical disinfectants, probably because they lack enzymes. Phenolic disinfectants are only weakly virucidal. Bacteria are killed in 50 per cent glycerol saline but this acts as a preservative for many viruses. The most active antiviral disinfectants are oxidizing agents such as hydrogen peroxide, potassium permanganate and hypochlorites. Organic iodine compounds are actively virucidal. Chlorination of drinking water kills most viruses but its efficacy is greatly influenced by the presence of organic matter. Some viruses (such as hepatitis virus, polioviruses) are relatively resistant to chlorination. The action of lipid solvents such as ether, chloroform and bile salts is selective, the enveloped viruses being sensitive and the naked viruses resistant to them. *The selective action is useful in the identification and classification of viruses.* Antibiotics active against bacteria are completely ineffective against viruses. This property is made use of in eliminating bacteria from clinical specimens by antibiotic treatment before virus isolation.

Properties of viruses

1. Small size cannot be viewed with a light microscope, pass through filters that retain bacteria range of size = 0.1–0.3 micrometers

2. Characteristic shapes — spherical (complex), helical, rod or polyhedral, sometimes with tails or envelopes. Most common polyhedron is the icosahedron which as 20 triangular faces.

3. Obligate intracellular parasites Viruses do not contain within their coats the machinery for replication. For this they depend upon a host cell and these accounts for their existence as obligate intracellular parasites. Each virus can only infect certain species of cells. This refers to the virus host range.

4. No builtin metabolic machinery Viruses have no metabolic enzymes and cannot generate their own energy.

5. No ribosomes Viruses cannot synthesize their own proteins. For this they utilize host cell ribosomes during replication. Features 4 and 5 accounts for the obligate intracellular parasitism of viruses.

6. Only one type of nucleic acid (contain either DNA or RNA (never both) as their genetic material. The nucleic acid can be single-stranded or double stranded).

7. Do not grow in size unlike cells; viruses do not grow in size and mass leading to a division process. Rather viruses grow by separate synthesis and assembly of their components resulting in production of a "crop" of mature viruses.

Classification of viruses

Table 10 — DNA viruses

<table>
<tr><th colspan="3">Double stranded DNA viruses</th><th>Single stranded nonenveloped DNA viruses</th><th>Complex enveloped DNA viruses</th></tr>
<tr><th>Enveloped</th><th colspan="2">Non-enveloped</th><td rowspan="3">PARVOVIRIDAE</td><td rowspan="3">POXVIRIDAE</td></tr>
<tr><td rowspan="2">HERPESVIRIDAE
HEPADNAVIRIDAE</td><th>Circular</th><th>Linear</th></tr>
<tr><td>PAPILLOMAVIRIDAE
POLYOMAVIRIDAE</td><td>ADENOVIRIDAE</td></tr>
</table>

Table 11 — RNA viruses

Single stranded RNA viruses (positive sense)			Single stranded nonenveloped DNA viruses	Complex enveloped DNA viruses
Enveloped		Non-enveloped	Enveloped	Non-enveloped
Icosaedral	Helical	Icosaedral	Helical	Icosaedral
FLAVIVIRIDAE TOGAVIRIDAE RETROVIRIDAE	CORONAVIRIDAE	PICORNAVIRIDAE CALICIVIRIDAE	PARAMYXOVIRIDAE RHABDOVIRIDAE FILOVIRIDAE BUNYAVIRIDAE ARENAVIRIDAE	REOVIRIDAE

Viral multiplication

The genetic information necessary for viral replication is contained in the viral nucleic acid. But viruses lack biosynthetic enzymes and depend on the synthetic machinery of the host cell for replication!

The viral multiplication cycle can be divided into six sequential phases

1. First stage in viral replication is called the **attachment (adsorption) stage** (see figure 40). Viruses attach to host cells by means of a complementary association between **attachment sites on the surface of the virus** and **receptor sites** on the host cell surface. This accounts for **specificity** of viruses for their host cells. Attachment sites on the viruses (usually called **virus receptors**) are distributed over the surface of the virus coat (capsid) or envelope, and are usually in the form of glycoproteins or proteins. Receptors on the host cell (called the **host cell receptors**) are generally glycoproteins imbedded into the cell membrane. Cells lacking receptors for a certain virus are resistant to it and cannot be infected. Attachment can be blocked by antibody molecules that bind to viral attachment sites or to host cell receptors. Since antibodies block the initial attachment of viruses to their host cells, the presence of these antibodies in the host organism is the most important basis for immunization against viral infections.

If the phase of adsorption can be bypassed, cells normally insusceptible to virus may be rendered susceptible to it. Thus, infectious nucleic acid (viroids) extracted from picornavirus can infect rodent cells, which are resistant to infection by the whole virus.

2. **Penetration stage** follows attachment. *Bacteria* possess rigid cell walls. Bacterial viruses cannot penetrate into bacterial cells and only the nucleic acid is introduced intracellularly by a complex mechanism. *Animal cells* do not have rigid cell walls and the whole virus can enter into them. Virus particles may be engulfed by a mechanism resembling phagocytosis, a process known as '*viropexis'*. Alternatively, in the case of the enveloped viruses, the viral envelope may fuse with the plasma membrane of the host cell and release the nucleocapsid into the cytoplasm.

3. **Uncoating** is the process of stripping the virus of its outer layers and capsid so that the nucleic acid is released into the cell. Most viruses enter the host cell in an engulfment process called *receptor mediated endocytosis* and actually penetrate the cell contained in a membranous structure called an endosome. Acidification of the endosome is known to cause rearrangements in the virus coat proteins which proba-

bly allow replacement of the viral core into the cytoplasm (liberation of nucleic acid). **With the most viruses, uncoating is effected by the action of lysosomal enzymes of the host cell.**

4. **Biosynthesis** includes synthesis not only of the *viral nucleic acid* and *capsid protein* but also of *enzymes* necessary in the various stages of vial synthesis, assembly and release. In addition, certain 'regulator proteins' are also synthesised which serve to shut down the normal cellular metabolism and direct the sequential production of viral components.

The site of viral synthesis depends on the type of virus. In general, most **DNA viruses** synthesise their nucleic acid in the **host cell nucleus**. The exceptions are the poxviruses, which synthesise all their components in the host cell cytoplasm. Most of **RNA viruses** synthesise all their components **in the cytoplasm**. Exceptions are orthomyxoviruses and some paramyxoviruses and retroviruses which are synthesised partly in the nucleus. **Viral protein** is synthesised **only in the cytoplasm**.

Biosynthesis consists essentially of the following steps:

1. **Transcription** of messenger RNA (mRNA) from the viral nucleic acid. The critical step in viral biosynthesis is the *transcription of mRNA from the viral nucleic acid*. Depending on the structure of their genome, viruses use **different strategies** for the transcription of mRNA. Viruses have been categorized into *six classes* by **Baltimore** (1970) *based on their replication mechanisms*.

Class 1: In the case of *fully double stranded DNA* viruses (most of viruses such as **adenoviruses, herpesviruses, papovaviruses**), the DNA enters the host cell nucleus and uses the host cell enzymes for transcription. The extracted DNA from these viruses is infectious! With **hepadnaviruses** which have a *partially double stranded DNA*, the duplex is completed by a viral DNA polymerase, inside the host cytoplasm. The mature DNA then moves into the nucleus, to be transcribed by *host transcriptases*. Extracted hepadnavirus DNA is not infectious!

Class 2: With *single stranded DNA* viruses (for example **parvovirus**), the DNA molecule moves into the host cell nucleus and is converted into the duplex form. Transcription is achieved by host enzymes.

Class 3: In **reoviruses**, the *double stranded RNA* is transcribed to mRNA by *viral polymerases*.

Class 4: Depending on the method of mRNA transcription, single stranded RNA viruses are classified into two categories. In the *positive strand* (plus strand, positive sense) *RNA* viruses, the viral RNA itself act as the mRNA. Viral RNA is infectious by itself and is translated *directly* into viral proteins in the host cell cytoplasm (**picornaviruses, togaviruses**).

Class 5: The *negative strand* (minus sense) *RNA* viruses (**rhabdoviruses, orthomyxoviruses, paramyxoviruses**) the RNA is "antisense", with *polarity opposite* to mRNA. They possess their *own RNA-polymerase* for mRNA transcription. Extracted nucleic acids from these viruses are not infectious!

Class 6: Retroviruses exhibit a unique replicative strategy. Their *single stranded RNA genome* is converted into an *RNA-DNA-hybrid* by the viral ***reverse transcriptase*** (RNA directed DNA polymerase) ***enzyme***. Double stranded DNA is then synthesised from the RNA-DNA-hybrid. The double stranded DNA form of the virus (provirus) is integrated into the host cell chromosome. This integration may lead to transformation of the cell and development of neoplasia.

2. **Translation** of the mRNA into ***"early proteins"***. These "early or nonstructural proteins" are *enzymes which initiate and maintain synthesis of virus components.* They may also induce shut down of host protein and nucleic acid synthesis!

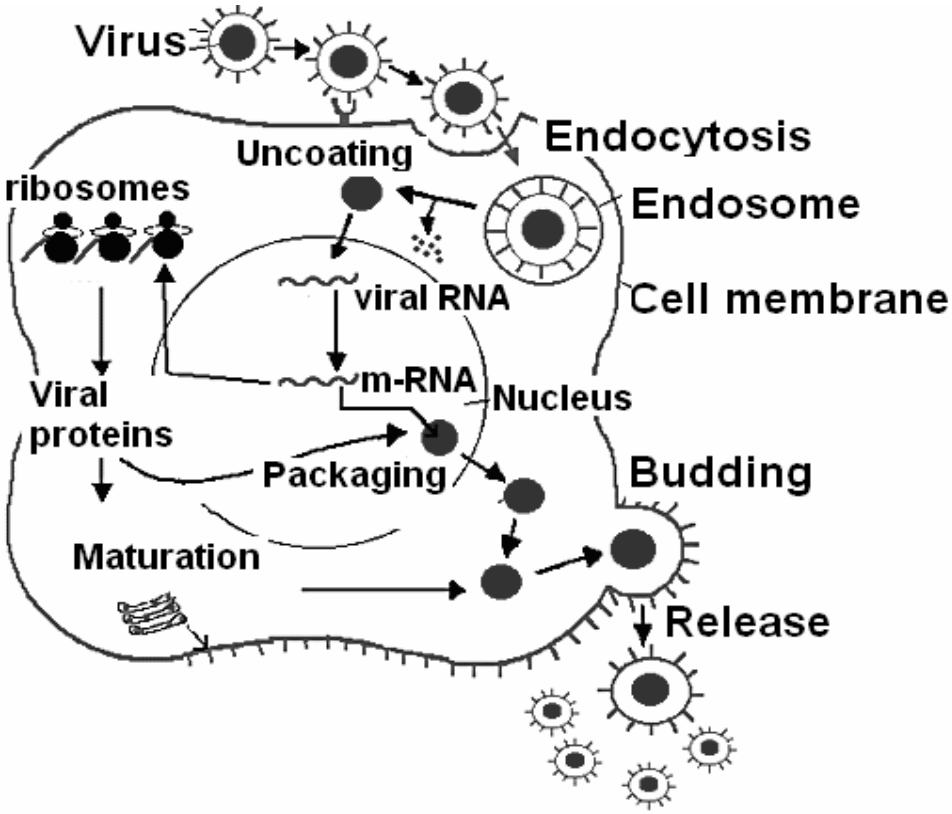


Figure 40 — The replication cycle of Influenza Virus

3. **Replication** of viral nucleic acid.

4. **Synthesis of "late" or structural proteins**, which are the components of daughter virion capsids.

5. **Maturation:** Assembly of *daughter virions* follows the synthesis of viral nucleic acid and proteins. Virion assembly may take place in the host cell nucleus or cytoplasm. Herpes- and adenoviruses are assembled in the nucleus, while picorna- and poxvirases are assembled in the cytoplasm. At this stage, the *nonenveloped viruses* are present intracellularly as fully developed virions but in the case of *enveloped viruses*, only the nucleocapsid is complete.

Envelopes are derived from the host cell membrane during the process of *budding*. The host cell membrane which becomes the envelope is modified by incorporation of *virus-specific antigens*. **Herpes viruses** assembled in the nucleus acquire their envelope *from the nuclear membrane* as they are released into the cytoplasm enclosed in a vesicle. **Myxovirases** bud from the cell surface and their envelope is formed by the modified *cytoplasmic membrane of the host cell*. The incorporation of viral antigen (hemagglutinin) on the cell membrane provides the cell with the property of hemadsorption.

6. **Release stage** is the final event in viral replication, and it results in the exit of the mature virions from their host cell. In the case of **bacterial viruses**, the release of progeny virions takes place by the *lysis* of the infected bacterium. However, in the case of **animal viruses**, release usually occurs *without cell lysis*. **Myxovirases** are released by a process of budding from the cell membrane over a period of time. The host cell is unaffected and may even divide, the daughter cells continuing to release virions. Progeny virions are released into the surrounding medium and may infect other cells.

From the stage of penetration till the appearance of mature daughter virions, the virus cannot be demonstrated inside the host cell. This period during which the virus seems to disappear or go 'under ground' is known as the **"eclipse phase"**. The time taken for a single cycle of replication is about 15–30 minutes for bacteriophages and about 15–30 hours for animal viruses. **A single infected cell may release a large number of progeny virions!** While this can be determined readily in bacteriophages (burst size), it is difficult to assess in the case of animal viruses that are released over a prolonged period.

How viruses cause disease

There are several possible consequences to a cell that is infected by a virus, and ultimately this may determine the pathology of a disease caused by the virus. **Lytic infections** (see figure 41) result in the destruction of the host cell.

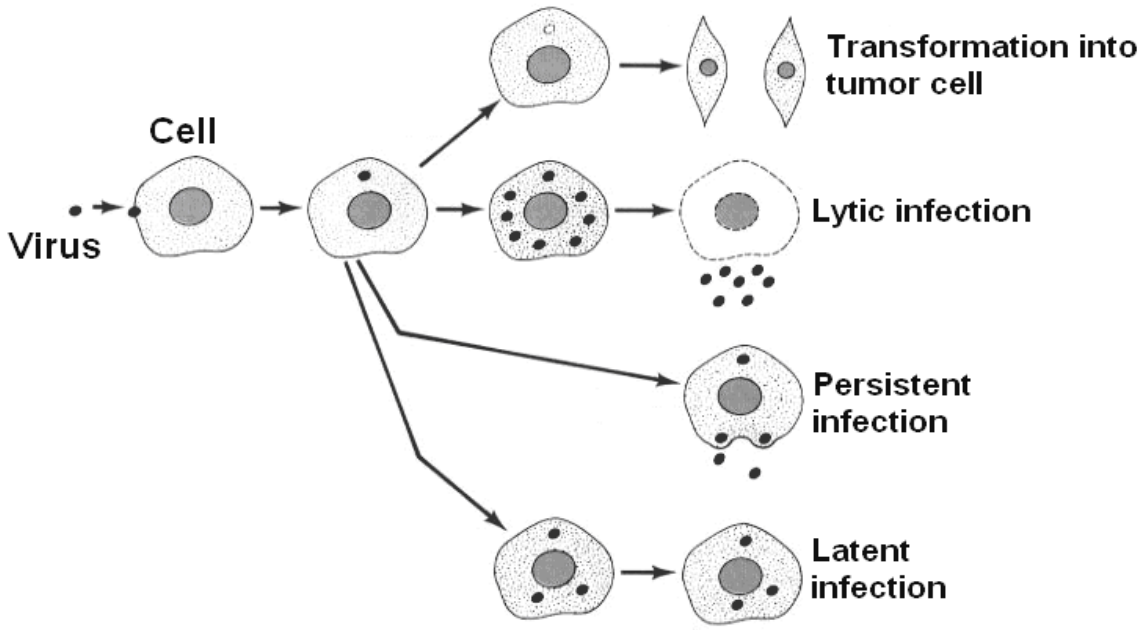


Figure 41 — The possible effects that animal viruses may have on the cells that they infect

Lytic infections are caused by *virulent viruses*, which inherently bring about the death of the cells that they infect. When enveloped viruses are formed by budding, the release of the viral particles may be slow and the host cell may not be lysed. Such infections may occur over relatively long periods of time and are thus referred to as **persistent infections**. Viruses may also cause **latent infections**. The effect of a latent infection is that there is a delay between the infection by the virus and the appearance of symptoms. Some animal viruses have the potential to change a cell from a normal cell into a tumor cell, the hallmark of which is to grow without restraint. This process is called **transformation.** Viruses that are able to transform normal cells into tumor cells are referred to as **oncogenic viruses**.

The vast majority of viral infections in humans are **inapparent** or **asymptomatic**. Viral pathogenesis is the abnormal situation and it is of no particular value to the virus, although it typically results in the multiplication of the viruses that can be transmitted to other individuals. For pathogenic viruses, there are a number of critical stages in replication which determine the nature of the disease they produce.

A proportion of daughter virions that are produced may not be infective. This is due to **defective assembly**. Such *'incomplete viruses*' are seen in large proportions when cells are infected with a high dose of the influenza virus.

Virus infection in some cells does not lead to production of infectious progeny. In such cells (*nonpermissive cells*), the vital components may be synthesised but maturation or assembly is defective, and either no release occurs or the progeny is noninfectious. This is known ap **abortive infection**. **Productive infection**: the cells are permissive for viral replication and virion progeny are released. **Restrictive infection**: the cell is transiently permissive and a few viruses are produced. Viral production then stop but the genome persists. Examples include Epstein Barr and herpes simplex.

Some viruses are genetically defective in that when they infect cells, they are unable to give rise to fully formed progeny. Example: hepatitis D virus and adeno-associated satellite virus which replicate only in the presence of their helper viruses — hepatitis B and adenoviruses respectively. Viruses which are genetically deficient and therefore incapable of producing infectious daughter virions without the helper activity of another virus are known as **"defective viruses"**. Infection of cells caused by nucleic acid alone is referred to as **transfection**.

Cultivation of viruses

As viruses are **obligate intracellular parasites**, they cannot be grown on any inanimate culture medium. Three methods are employed for the cultivation of ruses — *inoculation into* ***animals***, ***embryonated eggs*** or ***tissue cultures***.

Animal inoculation: The earliest method for the cultivation of viruses causing human diseases was inoculation into human volunteers. Due to the serious risk involved, human volunteers are used only when no other method is available and when the virus is relatively harmless. Monkeys were used for the isolation the poliovirus (1909). However, due to their cost and risk to handlers, monkeys find only limited application in virology. The use of white mice extended the scope of animal inoculation greatly. Mice are still the most widely employed animals in virology. Other animals such as guinea pigs, rabbits and ferrets are used in some situations.

The growth of the virus in inoculated animals may be indicated by death, disease or visible lesions. Disadvantages of animal inoculation are that immunity may interfere with viral growth and that animals often harbour latent viruses. Animal inoculation is also used for the study of pathogenesis, immune response, epidemiology and oncogenesis.

Embryonated eggs: The embryonated egg offers several sites for the cultivation of viruses. Inoculation on the *chorioallantoic membrane* produces visible lesions (pocks). Different viruses have different pock morphology. Inoculation into the *allantoic cavity* provides a rich yield of influenza. Inoculation into the *amniotic sac* is employed for the primary isolation of the influenza virus. *Yolk san* inoculation is used for the cultivation of some viruses, chlamydiae and rickettsiae.

Allantoic inoculation is employed for growing the influenza virus for vaccine production. Other chick embryo vaccines in routine use are the yellow fever and rabies vaccines.

Cell culture: Cultivation of bits of tissues and organs in vitro had been used by physiologists and surgeons for the study of morphogenesis and wound healing. The major obstacle to the development of tissue culture was the presence of *bacterial contamination*. It was only when antibiotics became available for the prevention of bacterial contamination that tissue culture became a routine laboratory method. The turning point which made tissue culture the most important method for the cultivation of viruses was the demonstration by Enders that poliovirus, till then considered a strictly neurotropic virus, could be grown in tissue culture of non-neural origin. Since then almost every human virus has been grown in tissue culture.

Three types of tissue cultures are available:

1. *Organ culture:* Small bits of organs can be maintained in vitro for days and weeks, preserving their original architecture and function. Organ cultures are useful for the isolation of some viruses which appear to be highly specialized parasites of certain organs. For example, the tracheal ring organ culture is employed for the isolation of coronavirus, a respiratory pathogen.

2. Explant culture: Fragments of minced tissue can be grown as 'explants' embedded in plasma clots. This was what was originally known as 'tissue culture'. This method is now seldom employed in virology. Adenoid tissue explant cultures were used for the isolation of adenoviruses.

3. Cell culture: This is the type of culture routinely employed for growing viruses. Tissues are dissociated into the component cells by the action of proteolytic enzymes and mechanical shaking. The cells are washed, counted and suspended in a growth medium. The essential constituents of the growth medium are physiologic amounts of essential aminoacids and vitamins, salts, glucose, and a buffering system. Antibiotics are added to prevent bacterial contaminants and phenol red as indicator. Such media will enable most cell types to multiply with a division time of 24–48 hours. The cell suspension is dispensed in bottles, tubes or Petri dishes. The cells adhere to the glass surface and on incubation, divide to form a confluent monolayer sheet of cells covering the surface within about a week.

Detection of virus growth in cell cultures: Virus growth in cell cultures can be detected by the following methods:

● **Cytopathic effect:** Many viruses cause morphological changes in cultured cells in which they grow. These changes can be readily observed by *microscopic examination* of the cultures. These changes are known as 'cytopathic effects' (CPE) and the viruses causing CPE are called **'cytopathogenic viruses'**. The CPE produced by different groups of viruses are characteristic and help in the *presumptive identification* of virus isolates. For example, measles virus produces syncytium formation; herpes virus causes discrete focal degeneration; adenovirus produces large granular clumps resembling bunches of grapes.

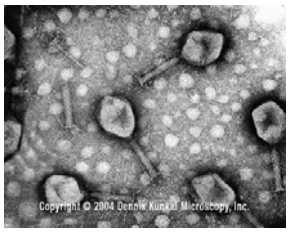
● **Metabolic inhibition**: In normal cell cultures, the medium turns acid due to cellular metabolism. When viruses grow in cell cultures, cell metabolism is inhibited and there is no acid production. This can be made out by the colour of the indicator (phenol red) incorporated in the medium.

● **Hemadsorption:** When hemagglutinating viruses (such as influenza and parainfluenza viruses) grow in cell cultures, their presence can be indicated by the addition of guinea pig erythrocytes to the cultures. If the viruses are multiplying in the culture, the erythrocytes will adsorb on the surface of cells. This is known as "**hemadsorption"**.

● **Interference:** The growth of a **noncytopathogenic virus** in cell culture can be tested by the subsequent challenge with a known cytopathogenic virus. The growth of the first will inhibit infection by the second virus by interference.

● **Transformation:** Tumour forming (oncogenic) viruses induce cell "transformation" and loss of contact inhibition, so that growth appears in a piled-up fashion producing "microtumours".

● **Immunofluorescence:** Cells from virus infected cultures can be stained by fluorescent antiserum and examined under the UV microscope for the presence of virus antigen. This gives positive results earlier than other methods and, therefore, finds wide application in diagnostic virology.



BACTERIOPHAGES

The definition of the term **bacteriophages** — bacterial viruses. Bacteriophages discovery — d'Herelle, 1917. *Nomenclature of phages is based on the name of the host which is sensitive to definite phage*. Bacteriophages (commonly abbreviated as phages) are viruses that infect bacteria. Twort (1915) described a degenerative change in staphylococcal colonies isolated from calf lymph, which could be transmitted serially by application of culture filtrates from the original growth. d'Herelle (1917) observed that the filtrates of feces cultures from dysentery patients induced transmissible lysis of a broth culture of a dysentery bacillus. He suggested that the lytic agent was a virus and gave it the name bacteriophage.

Phages occur widely in nature in close association with bacteria. They can be readily isolated from feces, sewage and other natural sources of mixed bacterial growth. Early hopes that phages could be used in the treatment of bacterial infections have not been fulfilled but these viruses have contributed much to microbiology. As phages could be grown easily on bacterial cultures, they provided the only *convenient model for the study of virus-host interactions* at the cellular and molecular levels before the development of cell culture techniques made similar studies with animal viruses possible. Phages play an important role in the transmission of genetic information between bacteria by the process of *transduction*. The presence of phage genome integrated with bacterial chromosomes confers on bacteria certain properties by a process known as **phage conversion**. Phages have been used as *cloning vectors* in genetic manipulations. The presence of high concentrations of phage particles in natural waters suggests that they may have a role in the control of bacterial populations in such environments. The specificity of the host range of phages is the basis of **phage typing methods**, by which bacteria can be identified and typed.

The main properties of bacteriophages

1. Obligative specificity.
2. They more tolerant to the high temperatures than viruses. Therefore bacteriophages can be isolated at 75 ºC.
3. Stability at the presence of chloroform and glycerin
4. Sensibility to the acid medium.
5. Stability to the antibiotics, disinfection and antiseptics.
6. Sensibility to the UV radiation, which have mutagenic influence.
7. Stability to the high pressure
8. The proteins of the phages cause the production of antibodies.

Morphology: Certain bacteriophages that infect E. coli, called the T even phages (T2, T4, T6), have been studied in great detail and traditionally serve as the prototypes in describing the properties of bacteriophages. T even phages have a *complex* and *characteristic* morphology. They are tadpole shaped, with a *hexagonal head* and a *cylindrical tail*. The head consists of a tightly packed core of nucleic acid (double stranded DNA) surrounded by a protein coat or capsid. The size of the head varies in different phages from 28 nm to 100 nm. The tail is composed of a hollow core,

a contractile sheath surrounding the core and a terminal base plate which has attached to it prongs, tail fibres or both (see figure 42). Though most bacteriophages have the morphology and structure described above, phages that are spherical or filamentous and possess single stranded DNA or RNA have also been identified.

Morphological types of phages

I type: only tail (filamentous);
II type: only head;
III type: short tail without protective cover and head;
IV type: long unretractile tail with protective cover and head;
V type: long retractile tail with protective cover and head.

Like all viruses, the bacteriophages carry only the genetic information needed for replication of their nucleic acid and synthesis of their protein coats. When phages infect their host cell, the order of business is to replicate their nucleic acid and to produce the protective protein coat. But they cannot do this alone. **They require precursors, energy generation and ribosomes supplied by their host cell!** Phages exhibit two different types of life cycle. In the **virulent** or **lytic cycle**, intracellular multiplication of the phage culminates in the *lysis of the host bacterium* and the release of progeny virions. In the **temperate** or **lysogenic cycle** the *phage DNA becomes integrated with the bacterial genome*, replicating synchronously with it, causing no harm to the host cell.

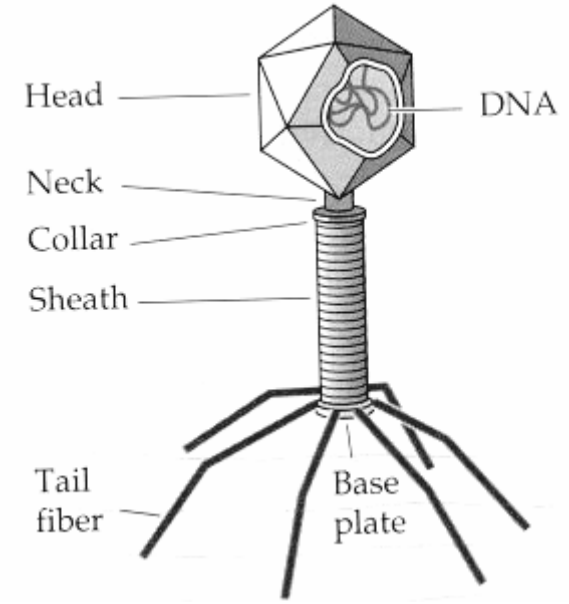


Figure 42 — Structure of bacteriophage

Lytic cycle

Replication of a virulent phage can be considered in the following stages 1) **adsorption**, 2) **penetration**, 3) **synthesis** of phage components, 4) **assembly**, 5) **maturation** and 6) **release** of progeny phage particles as illustrated in figure 43.

The first step in the replication of the phage in its host cell is called **adsorption**. Phage particles come into contact with bacterial cells by random collision. A phage attaches to the surface of a susceptible bacterium by its tail. Adsorption is a specific proc-

ess and depends on the presence of complementary chemical groups on the *receptor sites* of the bacterial surface and on the terminal base plate of the phage. Under optimal conditions, adsorption is a very rapid process, being complete within minutes.

The bacterial receptor sites may be situated in different layers of the cell wall or on surface structures (such as the Vi-antigen of the typhoid bacillus) or appendages (such as flagella or sex pili). Bacterial protoplasts, which are devoid of cell wall components, cannot adsorb phages and therefore will not be infected. *Host specificity of phages is determined at the level of adsorption*. Experimental infection by direct injection of phage DNA can be achieved even in bacterial strains that are insusceptible to infection by the whole phage. The infection of a bacterium by the naked phage nucleic acid is known as **transfection.**

Adsorption is followed by the **penetration of the phage nucleic acid** into the bacterial cell. The process of penetration resembles injection through a syringe. The phage DNA is injected into the bacterial body through the hollow core. Penetration may be facilitated by the presence on the phage tail of *lysozyme* which produces a hole on the bacterial wall for the entry of the phage core. The complex structure of the phage particle is required only for the injection of the nucleic acid into the host cell. The phage DNA alone is necessary for the initiation of the synthesis of *daughter phages*. After penetration, the empty head and tail of the phage remain outside the bacterium.

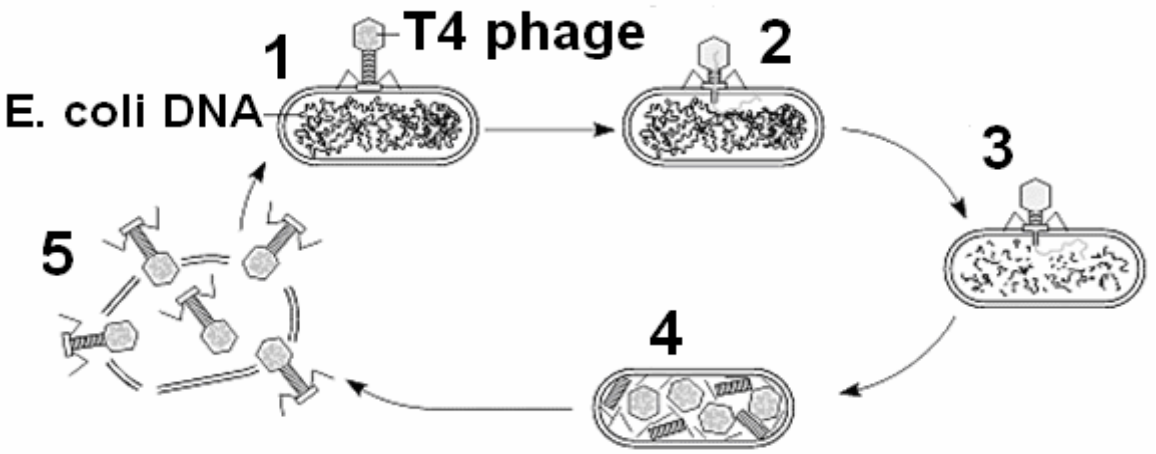


Figure 43 — The lytic cycle of phage

1. T4 phage sticks to specific receptor sites on surface of E. coli; 2. Sheath of tail contracts. Phage injects DNA into cell; 3. Empty capsid of phage left as "gnost" outside the cell; 4. Cell's metabolic machinery, directed by phage DNA, used to make copies of phage genome; 5. Phage directs production of enzymes that digests bacterial cell wall. Damaged wall, causes cell to burst, releasing phage particles.

Immediately after penetration of the phage nucleic acid, the **synthesis of the phage components** is initiated. The first products to be synthesised (called ***early proteins***) are the *enzymes* necessary for the building of the complex molecules peculiar to the phage. Subsequently, ***late proteins*** appear which include the *protein subunits of the phage head and tail*.

Phage DNA, head protein and tail protein are *synthesised separately* in the bacterial cell! The DNA is 'packaged' into the head and, finally, the tail structures are added. This assembly of the phage components into the mature infective phage particle is known as **maturation**.

Release of the mature progeny phages typically occurs by **lysis** of the bacterial cell. During the replication of the phage, the bacterial cell wall is weakened and it assumes a spherical shape. Phage enzymes act on the weakened cell wall causing it to burst or lyse resulting in the **release of mature daughter phages**.

Lysogenic cycle

Unlike **virulent phages** which produce lysis of the host cell, **temperate phages** enter into a *symbiotic relationship* with their host cells without destroying them. Following entry into the host cell, the *temperate phage nucleic acid becomes integrated with the bacterial chromosome*. The integrated phage nucleic acid is known as the **prophage**. *The prophage behaves like a segment of the host chromosome and replicates synchronously with it*. This phenomenon is called **lysogeny** and a bacterium that carries a prophage within its genome is called a **lysogenic bacterium**. Lysogenisation does not upset the bacterial metabolism.

The prophage confers certain *new properties* on the lysogenic bacterium. This is known as **lysogenic conversion** or **phage conversion**. This is *due to the synthesis of new proteins that are coded for by the prophage DNA.* An example is toxin production by the diphtheria bacillus, which is determined by the presence in it of the prophage beta. The elimination of the prophage abolishes the toxigenicity of the bacillus (see figure 44).

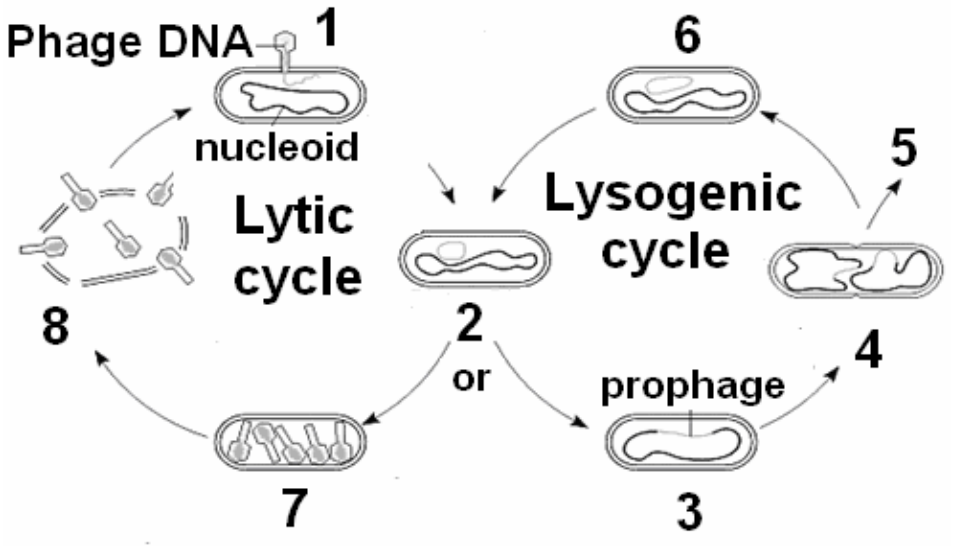


Figure 44 — The lysogenic cycle of phage

1. Phage attaches to host cell; injects DNA; 2. Phage DNA circularizes; 3. Phage DNA integrates into bacterial chromosome, becoming prophage; 4. Bactrrium reproduces, copying prophage and transmitting in to daughter cells; 5. Cell divisions produce bacteria with prophages; 6. Prophage exits bacterial chromosome, initiating lytic cycle; 7. New phage DNA and proteins are synthesized and assembled into phages; 8. Cell lyses, releasing phages.

During the multiplication of lysogenic bacteria, the prophage may become 'excised' from occasional cells. The excised prophage initiates lytic replication and the daughter phage particles are released, which infect other bacterial cells and render them lysogenic. This is known as **spontaneous induction of prophage**. While this is a *rare event*, all lysogenic bacteria in a population can be induced to shift to the lytic cycle by exposure to certain *physical* and *chemical agents*. Such inducing agents include UV rays and hydrogen peroxide.

A lysogenic bacterium is resistant to reinfection by the same or related phages. This is known as *superinfection immunity*. If a bacterium simultaneously adsorbs two related but slightly different DNA phage particles, both can infect and reproduce. On lysis, both types are released. When this occurs many of the progeny are observed to be recombinants.

Classification of the phages according to the spectrum of their infective effect:

◦ **Polyphages** (polyspesific phages): infect several species of bacteria;
◦ **Monophages** (monospesific phages): infect one species of bacteria;
◦ **Type phages** (type specific): infect only part of bacteria belonging to the same species (phage type).

Allocation of phages from environmental

1. Creation the conditions for the cultivation of the matrix bacteria for given phages.
2. Separation of the bacterial culture and phages cultures:
   a) Filtration with help of bacterial filters;
   b) Heating up to 75ºC;
   c) Treatment by chloroform.
3. Indication and identification of phages.
4. Determination of lytic activity.
5. Phage assimilation on the indicator (display) culture of sensitive microbe.
6. Quantitative calculation of phages – titration.

Phage assay

When a phage is applied on the lawn culture a susceptible bacterium, areas of clearing occur after incubation. These zones of lysis are called **plaques**. The size, shape and nature of plaques are characteristic for different phages. Since under optimum conditions single phage particle is capable of producing one plaque, plaque assay can be employed for titrating the number of viable phages in a preparation. As plaques are analogous to bacterial colonies, plaquing is al useful for the purification of phages (see figure 45).

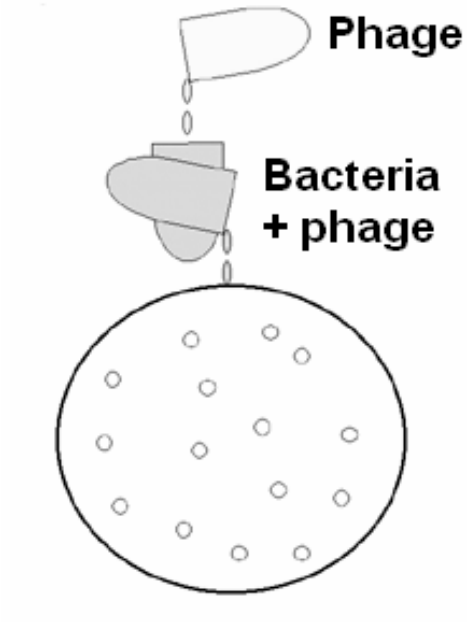


Figure 45 — Scheme of phage assay

Phage typing

The specificity of phage-bacterium interaction made use of in the identification and typing of bacteria. Phages exhibit different degrees of host specificity. The most important application of phage typing is for **intraspecies typing of bacteria,** as in the *phage typing of S. typhi and staphylococci*. Adapted phages, active only against fresh isolates possessing the Vi-antigen, are used for *phage typing of typhoid bacilli*. Staphylococcal phage typing is a pattern method, using a set of **standard phages.** A strain of Staphylococcus may be lysed by a number of phages and the phage type of a strain is designated by the numbers of the different phages that lyse it (see figure 46).

Phage preparations used for typing should be standardised by titration. Titration is carried out by applying serial dilutions of the phage preparation on a lawn culture of a susceptible strain and observing the lysis after incubation. The highest dilution of the phage preparation that just produces confluent lysis is known as the **routine test dose** (RTD).

The host specificity of bacteriophage is such that it is possible to outline different strains of individual species of bacteria on the basis of their susceptibility to various kinds of bacteriophage. In epidemiological studies, where it is important **to discover the source of a specific infection**, determining the phage type of the causative organism can be an important tool in solving the riddle. For example, if it can be shown that the phage type of *S. typhi* in a patient with typhoid fever is the same as the phage type of an isolate from a suspected carrier; chances are excellent that the two cases are epidemiologically related. Since all bacteria are probably parasitized by bacteriophages, it is theoretically possible, through research, to classify each species into strains or groups according to their phage type susceptibility. This has been done for *Staphylococcus aureus, Salmonella typhi,* and several other pathogens.

In bacteriophage typing, a suspension of the organism to be typed is swabbed over an agar surface. The bottom of the plate is marked off in squares and labeled to indicate which phage types are going to be used. To the organisms on the surface, a small drop of each phage type is added to their respective squares. After incubation, the plate is examined to see which phages were able to lyse the organisms.

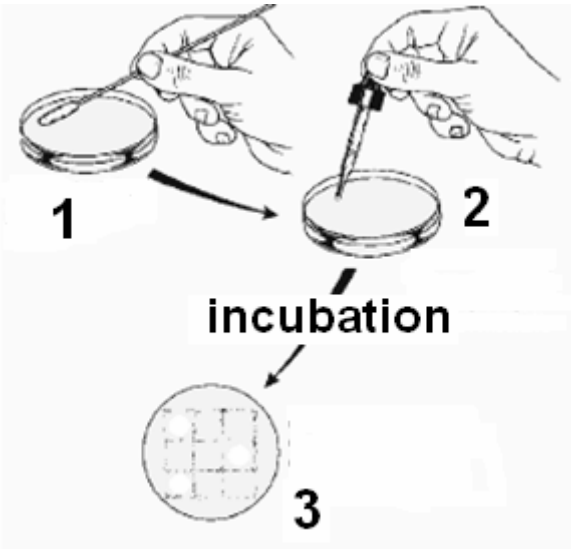


Figure 46 — Scheme of phage typing

1. Agar is swabbed with organisms to be typed. 2. Different phage types are added to swabbed surface of medium. 3. Bacteriophages that cause plague formation determine the phage type of the unknown.

Bacteriocins

Gratia (1925) observed the production of a highly specific antibiotic substance by one strain of E. coli which was active against another strain of the same species. The name **colicin** was given to such substances produced by E. coli and other members of the family Enterobactericeae. With the recognition that colicin-like substances are produced by several other bacteria also, the generic name **bacteriocin** was proposed for the *group of highly specific antibiotic-like substances produced by certain strains of bacteria which are active against other strains of the same or different species*. Bacteriocins are given specific names based on the bacterial species of origin, for example colicins from E .coli and diphthericins from C. diphtheriae.

Bacteriocins and phages resemble each other in a number of respects. Both adsorb on the surface of susceptible bacterial cells on specific receptor sites some of which may be the same for phages and bacteriocins. Under the electron microscope, some bacteriocins appear like the tail structures of phages. They may be considered products of defective phage genomes, able to code only for parts of phage particles.

The synthesis of bacteriocins (colicins) is determined by the presence in bacteria of **colicinogenic factors** (Col-factors). Col-factors are *plasmids* and can be transmitted from cell to cell by *conjugation* or *transduction*.

Biological role of colicins: 1) decrease of the density of bacterial population under the conditions when the nutrient media got exhausted importance for medicine; 2) participate in normalization of natural microbiocenosis of intestinal habitants.

Properties of bacteriocins:

1. Bacteriocins resemble the antibiotics with narrow spectrum of action.
2. Bacteriocins are causing the destruction of target cells.
3. Bacteriocins inhibit the synthesis of DNA, RNA and proteins.
4. After the releasing of bacteriocins the cell will die.
5. Bacteria producing the bacteriocins will be resistant to the action of the same bacteriocins from the outside.

A cell producing a bacteriocin is immune to it but may be sensitive to other bacteriocins. Bacteriocins have a very specific activity on bacteria, being capable of killing some but not all strains of a species. The specificity is made use of in typing certain species such as *Sh. sonnei, Proteus sp., Ps. aeruginosa*. Bacteriocins kill susceptible cells without lysing them.

While **phage typing schemes** are generally based on the sensitivity of the test strains to the lytic action of phages, **bacteriocin typing schemes** depend on the ability of bacteriocins produced by the test strain to kill standard indicator strains of bacteria. The usual method of bacteriocin typing employs the **plate diffusion technique**. The test bacterium is inoculated as a broad streak on the centre of a culture medium, the bacterial growth is scraped off and the remaining cells killed by exposure to chloroform vapour. Standard indicator strains of bacteria are then streaked at right angles to the original inoculum. After incubation, the pattern of inhibition of the indicator strains represents the bacteriocin type of the test bacterium.

GENETICS OF BACTERIA

The genetic material of bacteria and plasmids is **DNA**. Bacterial viruses (bacteriophages or phages) have DNA or RNA as genetic material. The two essential functions of genetic material are replication and expression. Genetic material must replicate accurately so that progeny inherit all of the specific genetic determinants (the genotype) of the parental organism. Expression of specific genetic material under a particular set of growth conditions determines the observable characteristics (phenotype) of the organism. Bacteria have few structural or developmental features that can be observed easily, but they have a vast array of biochemical capabilities and patterns of susceptibility to antimicrobial agents or bacteriophages. These latter characteristics are often selected as the inherited traits to be analyzed in studies of bacterial genetics.

Genetic information in microbes

The central dogma of molecular biology:

DNA → m-RNA → PROTEIN

The central dogma concerns the flow of biological information: DNA is a self-replicating molecule containing genetic information that can be *transcribed* into an RNA message that can be *translated* into a polypeptide (protein).

Transfer of the information to RNA using a DNA template is called **transcription**, and the molecule that encodes for one or more polypeptides (proteins) is called **messenger RNA** (mRNA). During **translation**, this genetic code in mRNA is read and converted into protein by means of the protein synthesizing machinery, which consists of ribosomes, tRNA, amino acids and a number of enzymes. A single mRNA often contains more than one coding region, separated during translation into distinct polypeptides. Also, in procaryotes transcription and translation are "*coupled*", meaning that they occur simultaneously.

During **replication**, as the DNA is unwound and the two strands are separated, the newly synthesized strand that is oriented 5'→3' is called the **leading strand**, and the new strand that is oriented 3'→5' is called the **lagging strand**. During DNA replication DNA is duplicated, producing two double helices.

Four nucleic acid bases are found in DNA, adenine (A), guanine (G), cytosine (C) and thymine (T). The double helix of DNA has a complementary and antiparallel structure. One chain ends in a 5'-phosphate and the opposite chain ends in a 3'-hydroxyl.

The **Genetic code** allows for correspondence between triplets of bases in DNA and the amino acid sequence of a polypeptide (protein) written or expressed in terms of RNA triplets as compared to DNA triplets because it is with messenger RNA that the translation process occurs.

In fact, the genetic code is not completely universal — there are minor differences in the codons which are recognised or used by different groups of organisms, e.g. animals, plants, bacteria. However, this rarely presents a difficulty expressing foreign genes, or if it does, the means exist to modify particular codons to alternatives coding for the same amino acids which will be recognised by the host organism.

The sum total of the genes that make up the genetic apparatus of the cell (*genome*) establishes its **genotype**, which is the hereditary constitution of the cell that is transmitted to its progeny. The genotype includes the complete genetic potential of the cell, all of which may or may not be expressed in a given environmental situation.

The **phenotype** is the physical expression of the genotype in a given environment. It means that a cell may exhibit different phenotypic appearances in different situations; for example, the typhoid bacillus is normally flagellated but when grown in phenol agar, the flagella are not synthesized. This only a phenotypic variation determined by the environment is reversed when subcultured from phenol agar into broth.

Organization of genetic material in bacteria

Genetic material in bacteria is containing in **nucleoid** and **extrachromosomal genetic material** which may be:

1. **Autonomous** (replication is independent) — **plasmids**;

2. **Nonautonomous (**replication is simultaneously with nucleoid or plasmid — **IS-elements, transposons** and **temperate bacteriophages**).

The amount of DNA in the genome determines the maximum amount of information that it can encode. Most bacteria have a *haploid* genome, a single chromosome consisting of a **circular, double stranded DNA molecule**. However linear chromosomes have been found in Gram-positive Borrelia and Streptomyces. The chromosome of E coli has a contour length of approximately 1.35 mm, several hundred times longer than the bacterial cell, but the DNA is super coiled and tightly packaged in the bacterial nucleoid. The time required for replication of the entire chromosome is about 40 minutes, which is approximately twice the shortest division time for this bacterium. DNA replication must be initiated as often as the cells divide, so in rapidly growing bacteria a new round of chromosomal replication begins before an earlier round is completed.

Nucleoid (***bacterial chromosome***) codes vital information. **Extra-chromosomal factors** of heredity code the information which is not important for life. **Plasmids** are replicons that are maintained as *discrete, extrachromosomal genetic elements in bacteria* (**autonomous factors of heredity in bacteria**). They are usually much smaller than the bacterial chromosome. Plasmids replicate *independently* of the chromosome. Most plasmids are super coiled, circular, double-stranded DNA molecules, but linear plasmids have also been demonstrated in Borrelia and Streptomyces. Closely related or identical plasmids demonstrate *incompatibility*; they cannot be stably maintained in the same bacterial host.

Conjugative plasmids code for functions that promote transfer of the plasmid from the donor bacterium to other recipient bacteria (contain special transfer-operon or *tra-operon*), but **nonconjugative plasmids** do not (without tra-operon). Conjugative plasmids that also promote transfer of the bacterial chromosome from the donor bacterium to other recipient bacteria are called **fertility plasmid**. *Large plasmids* are often *conjugative;* code for all functions required for their replication and divide themselves among daughter cells during cell division in a manner similar to the bacterial chromosome. *Plasmids smaller* usually are *nonconjugative*, depend on their bacterial host to provide some functions required for replication and are distributed randomly between daughter cells at division.

Functions of plasmids:

1. *Regulatory* — plasmids compensate infringements of the function of DNA of bacterial nucleoid.

2. *Coding* — plasmids introduce new information into the genotype of bacteria.

Possible location of plasmids in the bacterial cell:

1. Autonomous (in cytoplasm);

2. Integrated (inserted into the nucleoid).

Character of the control of the replication of plasmid DNA by the nucleoid:

1. *Strict control* (replication synchronically with nucleoid and produce 1–2 copies per one bacterial cell — large plasmids).

2. *Weak control* (replication more frequently then nucleoid and produce 10–30 copies per bacterial cell — small plasmids).

Classification of plasmids

● Plasmids that determine *multiple resistance to antibiotics* and other medical preparations are often called **R-plasmids**.

● Representative toxins encoded by plasmids include heat-labile and heat-stable *enterotoxin*s of E coli, exfoliative toxin of Staphylococcus aureus, and tetanus toxin of *Clostridium tetani* (**Ent-plasmid**).

● **Hly-plasmids** control the synthesis of *hemolysin* (enzyme of invasiveness).

● **F-plasmids** (fertility factor) provide conjugation between mating bacteria.

● **Plasmids of bacteriocinogenicity (colicinogenic factor or Col-factor)**: several strains of coliform bacteria produce **colicins** — antibiotic-like substances which are specifically and selectively lethal to other enterobacteria. Colicin is example of bacteriocins. Thus, **bacteriocins** — antibiotic-like substances are causing the destruction of the closely related species of bacteria.

● **Biodegradative plasmids** provide of bacterial stability to different physical factors and utilization of harmful organic and inorganic compounds.

A lot of plasmids are being *eliminated* under the influence of different inductors (UV, temperature, stains, mutagens, etc.) or spontaneously!

F-plasmid is a transfer factor that contains genetic information necessary for the synthesis of the sex pili and for self-transfer during conjugation (contains only *tra-operon,* any other genes are not presented).

Location of F-plasmid in the bacterial cell

F+-cells: carry a plasmid called the *fertility factor* or *F-factor*; they are genetic **donors** (**male cells**) in conjugation matings. **F^--cells:** lack the F factor; act as genetic **recipients (female cells)** in matings. *Cells carrying the F+-factor have no distinguishing features other than their ability to mate with F^--cells and render them F+-state!* This is illustrated in figure 47.

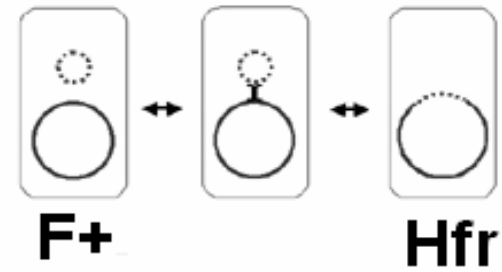


Figure 47 — Location of F-plasmid

Hfr-cells (high frequency recombination): F-factor is integrated into the bacterial chromosome. Hfr-cells act as **genetic donors** in conjugation and can transfer part or the bacterial chromosome to F^--recipients with high frequency. This conversion of an F+-cells into the Hfr-state is reversible. When the factor reverts from the integrated state to the free state, it may sometimes carry with it some chromosomal genes. Such cells are called F prime or F`-cells.

F`-cells: F-factor is extrachromosomal but has attached to it a segment of chromosomal genes.

R-plasmids are plasmids coding *multiple resistance to antibiotics*. Composition: operon (**resistance determinant**) and ***tra-operon*** (transfer factor or **RTF** – resistance transfer factor) which is responsible for conjugational or other transfer. The ways of transmission: *transduction* (with Gram-positive bacteria) and *conjugation* (with Gram-negative bacteria).

Plasmids of bacteriocynogenity (e. g. Col-plasmids of E.coli) are plasmids coding synthesis of bacteriocins. Composition: contain genes coding synthesis of bacteriocin and tra-operon. Peculiarities of these plasmids:

◦ Rarely integrated into the nucleoid;

◦ Usually exist in repressed state;

◦ After the plasmid derepression bacterial cell synthesizes bacteriocins and dies after that (*potentially lethal plasmid*).

Practical importance of bacteriocinogenicity:

1. Antagonistic relations in bacterial world (bacteriocinogenicity is *factor of resistance to the infections* in nonpathogenic bacteria and *factor of pathogenicity* in pathogenic bacteria);

2. Ability to produce bacteriocins is hereditary feature of defined bacterial strain (use in identification of unknown bacteria).

Mobile genetic elements

Certain structurally and genetically discrete segments of DNA have been identified that have the ability to move around between chromosomal and extrachromosomal DNA molecules within cells.

Transposons ("jumping genes") are segments of DNA that can move from one site in a DNA molecule to other target sites in the same or a different DNA molecule. The process is called **transposition** and occurs by a mechanism that is independent of generalized recombination. Transposons are important genetic elements because they cause *mutations*, mediate *genomic rearrangements (mutative variability)*, acquire *new genes* and contribute to their *spreading* within bacterial populations (see figure 48).

Transposons **are not self-replicating genetic elements**, however, and they must integrate into other replicons to be maintained stably in bacterial genomes! During transposition a short sequence of target DNA is duplicated and the transposon is inserted between the directly repeated target sequences. The length of this short duplication varies, but is characteristic for each transposon. Two types of transposition are recognized. Excision of the transposon from a donor site followed by its insertion into a target site is called **nonreplicative transposition**. If the transposon at a donor site is replicated and a copy is inserted into the target site, however, the process is called **replicative transposition**.

By transposition a segment of DNA can be transferred from a molecule to another molecule that has no genetic homology with the transposable element and donor DNA. In this difference from recombination.

Location of transposon in bacterial cell: 1) integrated into the nucleoid (replicate simultaneously; 2) autonomous (when located in cytoplasm they take circular shape and do not replicated themselves).

Figure 48 — Structure of transposon

Each transposon contains **special terminated sequences** by which transposon could be distinguished from other DNA fragments (*markers of transposon*); **genes of transposition** and **genes** coding synthesis of toxins, enzymes participating in the development of resistance to antibiotics and other properties.

IS-elements (small transposons) inserts of nucleotide sequences (usual their size is about 1 000 pairs of nucleotides). Differences in compare with transposons: 1) contain only genes coding **transposition**; 2) never found in autonomous state.

Functions of IS-elements:

1. Coordination of counteraction of extra-chromosomal factors of heredity: between each others and with bacterial chromosome to provide their recombination
2. Regulatory (regulation of the transcription of genes by the mechanism of their "switch on/switch off").
3. Induction of the mutations (inversions, duplications which take place by involving about 10 pairs of nucleotides).

Variability in bacterial world

Table 12 — Types of bacterial variability

Modification variability (*phenotypic* or *nonheritable*)	**R-S-dissociation (*specific variability in bacteria*)**	**Genetic variability (*genotypic* or *heritable*)**	
Changes affecting only the phenotype of bacteria, do not accompanied by changes of DNA structure, not stable and usually could be lost very quickly (temporary). May be morphological, biochemical, etc. **Mechanism:** *Inducible* synthesis of specific enzymes in presence if suitable substrate.	Appearance of *R-shaped colonies* in pure bacterial culture which normally form *S-shape colonies*: phenotypic manifestation of the change of some properties of the bacterial cells. This type may be referred to modification or mutation variability.	**Mutation variability**	**Recombinative variability (**in the basis is **recombination)**
		Changes which occur in the primary structure of DNA molecule. The result is loss or change of one or several hereditary features which will be inherited by the next generations	Changes in DNA structure occurring as a result of integration of the part of DNA of the recipient cell into the DNA of the donor cell Forms: *Conjugation* *Transformation* *Transduction* *Lysogenic conversion*

Mutations in bacteria

Mutation is a random, undirected, heritable variation caused by an alteration in the nucleotide sequence at some point of DNA of the cell.

If a gene that encodes for a specific protein is mutated, it may result in a change in the sequence of amino acids comprising the protein. The activity of the protein may be altered. Frequency of naturally occurring (spontaneous) mutation varies from 10^{-6} to 10^{-9}.

Mutations result from two underlying causes:

1. **Substitutions** — one base is substituted in the DNA for another base. Incorrect base pairing results from the change of a single nucleotide base. Substitutions usually result in "point mutations".

2. **Insertions** or **deletions** of one or more bases — may result in the addition or deletion of one or more amino acids to the growing protein, or more likely result in a "frame-shift" mutation.

Multiple mutations cause extensive chromosomal rearrangements. A **missense mutation** is one in which the triplet code is altered so as to specify an aminoacid different from that normally located at a particular position in the protein. Deletion of a nucleotide within a gene may cause premature polypeptide chain termination by generating a nonsense codon, i.e., **nonsense mutation**.

Classification mutations according to their direction:

- Direct (loss or change of the property).
- Reverse (restoration of the property).

1. True (when restoration of genotype and phenotype takes place).
2. Suppressive (when we see restoration only of phenotype).

Transversion is substitution of a purine for a pirimidine and vice versa in base pairing. **Suppressor mutation** is reversal of a mutant phenotype by another mutation at a position on the DNA distinct from that of the original mutation.

All genes are sensitive to mutational events but not all mutations are expressed. Some mutations involve vital functions, and such mutants are nonviable (**lethal mutations**). A type of lethal mutation is **conditional lethal mutation**. These mutants may be able to live under certain conditions (permissive conditions).

Classification mutations by the occurring mechanism:

1. Spontaneous (difficult or not possible to find the effect of curtain factor — mutagen);
2. Inductive (produced in the experiment when certain known mutagen is applied).

Classification mutations according to changes in phenotype:

1. Morphological;
2. Biochemical;
3. Physiological.

Mutation and selection

Mutations are heritable changes in the genome. Spontaneous mutations in individual bacteria are rare. Some mutations cause changes in phenotypic characteristics; the occurrence of such mutations can be inferred from the effects they produce. In microbial genetics specific reference organisms are designated as **wild-type**

strains, and descendants that have mutations in their genomes are called **mutants**. Thus, **mutants are characterized by the inherited differences between them and their ancestral wild-type strains**.

Detection of mutant phenotypes

Selective and *differential media* are helpful for isolating bacterial mutants. Some *selective* media permit particular mutants to grow, but do not allow the wild-type strains to grow. Rare mutants can be isolated by using such selective media. *Differential* media permit wild-type and mutant bacteria to grow and form colonies that differ in appearance.

Selective media for Lac^- mutants of E coli can be made by incorporating chemical analogs of lactose that are converted into toxic metabolites by Lac+ bacteria but not by Lac- mutants. The Lac- mutants can then grow on such media, but the Lac+ wild-type bacteria are killed. The mutation rate in bacteria is determined by the accuracy of DNA replication, the occurrence of damage to DNA, and the effectiveness of mechanisms for repair of damaged DNA.

For a particular bacterial strain under defined growth conditions, the mutation rate for any specific gene is constant and is expressed as the probability of mutation per cell division. In a population of bacteria grown from a small inoculum, the proportion of mutants usually increases progressively as the size of the bacterial population increases.

An excellent way to determine the ability of organisms to produce mutants that are resistant to antibiotics is to grow them on a **gradient plate** of a particular antibiotic. Such a plate consists of two different layers of media: a bottom layer of plain nutrient agar and a top layer of nutrient agar with the antibiotic. Since the antibiotic is only in the top layer, it tends to diffuse into the lower layer, producing a gradient of antibiotic concentration from low to high. Any colonies that develop in the high concentration area will be antibiotic-resistant mutants.

To determine whether or not such a colony exists on a plain agar plate having 500 to 1,000 colonies could be a laborious task. One would have to transfer organisms from each colony to a medium containing streptomycin. This is somewhat self-defeating, too, in light of the low incidence of mutations that occur. Many thousands of the transfers might have to be made to find the first mutant. Fortunately, we can resort to **replica plating** to make all the transfers in one step. Figure 49 illustrates the procedure. In this technique a velveteen-covered colony transfer device is used to make the transfers.

Note in figure 49 that organisms are first dispersed on nutrient agar with a glass spreading rod. After incubation, all colonies are transferred from the nutrient agar plate to two other plates: first to a nutrient agar plate and second to a streptomycin agar plate. After incubation, streptomycin-resistant strains are looked for on the streptomycin agar.

Induced mutation is caused by **mutagens,** substances that cause a much higher rate of mutation.

Chemical mutagens generally work in one of three ways:

- Some chemical mutagens, such as nitrous acid and nitrosoguanidine work by causing chemical modifications of purine and pyrimidine bases that alter their hydrogen-bonding properties. For example, nitrous acid converts cytosine to uracil which then forms hydrogen bonds with adenine rather than guanine.

• Other chemical mutagens function as **base analogs**. They are compounds that chemically resemble a nucleotide base closely enough that during DNA replication, they can be incorporated into the DNA in place of the natural base. Examples include 2-amino purine, a compound that resembles adenine.

• Still other chemical mutagens function as **intercalating agents**. Intercalating agents are planar three-ringed molecules that are about the same size as a nucleotide base pair. During DNA replication, these compounds can insert it intercalate between adjacent base pairs thus pushing the nucleotides far enough apart that an extra nucleotide is often added to the growing chain during DNA replication. An example is ethidium bromide.

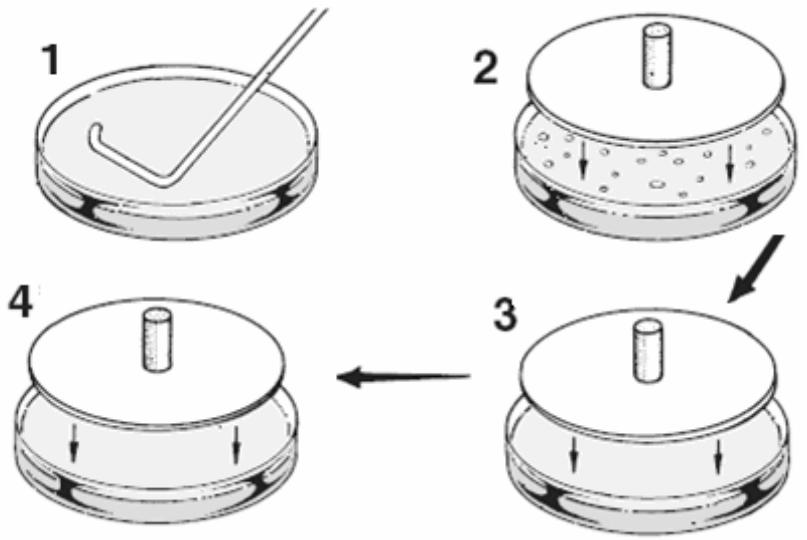


Figure 49 — Replica plating technique

1. Organisms are spread oover nutrient agar with the steril bent glass rod.
2. After incubation, colonies are picked up with velveteen colony carrier.
3. Streptomycin agar is inoculated with same carrier in same manner.
4. Nutrient agar is inoculated by lightly pressing the carrier onto it.

Certain types of radiation can also function as mutagens:

• **Ultraviolet Radiation.** The ultraviolet portion of the light spectrum includes all radiations with wavelengths from 100 nm to 400 nm. It has low wave length and low energy. The microbicidal activity of ultraviolet (UV) light depends on **the length of exposure**: the longer the exposure the greater the cidal activity. It also depends on the **wavelength of UV used**. The most cidal wavelengths of UV light lie in the 260–270 nm range where it is absorbed by nucleic acid.

In terms of its mode of action, UV light is absorbed by microbial DNA and causes adjacent thymine bases on the same DNA strand to covalently bond together, forming what are called **thymine-thymine dimers**. As the DNA replicates, nucleotides do not complementary base pair with the thymine dimers and this terminates the replication of that DNA strand. However, **most of the damage from UV radiation actually comes from the cell trying to repair the damage to the DNA by a process called SOS repair**.

In very heavily damaged DNA containing large numbers of thymine dimers, a process called SOS repair is activated as kind of a last ditch effort to repair the DNA. In this process, a gene product of the SOS system binds to DNA polymerase allow-

ing it to synthesize new DNA across the damaged DNA. However, this **altered DNA polymerase loses its proofreading ability** resulting in the synthesis of DNA that it self now contains many misincorporated bases.

• **Ionizing Radiation.** Ionizing radiation, such as **X-rays and gamma rays**, has much more energy and penetrating power than ultraviolet radiation. It ionizes water and other molecules to form radicals (molecular fragments with unpaired electrons) that can **break DNA strands and alter purine and pyrimidine bases**.

Chemicals that are carcinogenic for animals are often mutagenic for bacteria, or can be converted by animal tissues to metabolites that are mutagenic for bacteria. Standardized tests for mutagenicity in bacteria are used as *screening procedures* to identify environmental agents that may be carcinogenic in humans. Mutation may affect any gene and hence may modify and characteristic of the bacterium. Mutants may vary in properties such as nutritional requirements, biochemical reaction, antigenic structure, morphological features, colony form, drug resistance, virulence and host range. The practical important of bacterial mutation lies mainly in the field of drug resistance and the development of live vaccines.

SR-dissociation

Mechanism: insertion mutation resulting in the loss of the genes controlling synthesis of carbohydrates chains which are necessary for the formation of LPS component of the cell wall.

Biological importance:

1. Bacteria producing *R-shaped colonies* are more resistant to unfavorable physico-chemical factors of the external environment.
2. Bacteria producing *S-shaped colonies* are more resistant to phagocytosis and to the effect of antibodies.

SR-dissociation significantly complicates isolation and identification of pure culture!

Table 13 — Comparison of S-colonies and R-colonies

S-colony	R-colony
Smooth	Rough
Usual virulent	Usual nonvirulent (exception B. anthracis, C.diphtheria. Y.pestis)
Capsule and flagella are present	Capsule and flagella are not present
Sensitive to bacteriophages	Less sensitive to bacteriophages
Biochemically active	Biochemically less active
Complete set of antigens	Incomplete set of antigens

Recombinative variability in bacteria

Transformation is gene transfer resulting from the uptake by a recipient cell of naked DNA from a donor cell. Certain bacteria (*e.g.* Bacillus, Neisseria) can take up DNA from the environment and the DNA that is taken up can be incorporated into the recipient's chromosome. Pieces of DNA involved in transformation may carry about 10–50 genes.

Factors affecting transformation:

1. **DNA size state**: Double stranded DNA of works best. Thus, transformation is sensitive to nucleases in the environment.

2. **Competence of the recipient**: Some bacteria are able to take up DNA naturally. However, these bacteria only take up DNA a particular time in their growth cycle when they produce a specific protein called a *competence factor*. At this stage the bacteria are said to be *competent*. Other bacteria are not able to take up DNA naturally. However, in these bacteria competence can be induced in vitro by treatment with chemicals (*e.g.* $CaCl_2$).

Steps in transformation

1. **Uptake of DNA**: Uptake of DNA by Gram-positive and Gram-negative bacteria differs. In Gram-positive bacteria the DNA is taken up as a *single* stranded molecule and the complementary strand is made in the recipient. In contrast, Gram-negative bacteria take up double stranded DNA.

2. **Homologous Recombination**: After the donor DNA is taken up, a reciprocal recombination event occurs between the chromosome and the donor DNA. This recombination requires homology between the *donor DNA* and the chromosome and results in the substitution of DNA between the recipient and the donor as illustrated in Figure. Because of the requirement for homology between the donor and host DNA, only DNA from closely related bacteria would be expected to successfully transform, although in rare instances gene transfer between distantly related bacteria has been shown to occur.

3. **Significance:** Transformation occurs in nature and it can lead to increased virulence. In addition transformation is widely used in recombinant DNA technology.

Transduction is the transfer of genetic information from a donor to a recipient by way of a *bacteriophage*. **Bacteriophages** are viruses that parasite bacteria and consist of nucleic acid core and a protein coat. Not all phages can mediate transduction. In most cases gene transfer is between members of the same bacterial species. However, if a particular phage has a wide host range then transfer between species can occur. The ability of a phage to mediated transduction is related to the life cycle of the phage.

Types of transduction

● **Generalized transduction** is transduction in which potentially *any* bacterial gene from the donor can be transferred to the recipient. Phages that mediate generalized transduction generally *breakdown* host DNA into smaller pieces and package their DNA into the phage particle by a "head-full" mechanism. Occasionally one of the pieces of host DNA is randomly packaged into a phage coat. Thus, any donor gene can be potentially transferred. If a recipient cell is infected by a phage that contains donor DNA, *donor DNA* enters the recipient. ***Bacteriophage fulfills only transport function for donor genes during transduction!*** In the recipient a generalized recombination event can occur which substitutes the donor DNA and recipient DNA.

● **Specialized transduction** is transduction in which only *certain* donor genes can be transferred to the recipient. Different phages may transfer different genes but an individual phage can only transfer certain genes. For example, prophage lambda is inserted in the bacterial chromosome only between the genes determining galactose utilization and biotin synthesis. Formation of the *transductional phage* is effected by discharging of a prophage together with the chromosomal genes of a cell-donor (*defective phage*). Then this *phage DNA* and *donor genes* must be incorporated in genome of recipient cell! Transduction is not confined to transfer of

chromosomal DNA. Plasmids may be also be transduced. The plasmids determining penicillin resistance in staphylococci are transferred by transduction. Any group of bacteria for which bacteriophage exists can be subject to transduction.

● **Abortive transduction** occurs when the new DNA does not integrate into the chromosome — may affect the phenotype but is not replicated and is eventually lost.

● **Transposition and insertional mutagenesis:** A few temperate phages act as transposons and move from site to site in the bacterial chromosome. New insertions may result of the inactivation of bacterial genes (promoters or coding sequences).

Lysogenic conversion: Bacteriophages exhibit two types of life cycle. In the *virulent* or *lytic* cycle, large numbers of progeny phages are built up inside the host bacterium, which ruptures to release them. In the *temperate* or *nonlytic* cycle, the host bacterium is unharmed. The phage DNA becomes integrated with the bacterium chromosome as the **prophage**, which multiplies synchronously with the host DNA and is transferred to the daughter cells. This process is called **lysogeny** and bacteria carrying the prophages are called **lysogenic bacteria**.

In lysogenic bacteria, the prophage behaves as an additional segment of the bacterial chromosome, coding for new characteristics. This process by which the prophage DNA confers genetic information to a bacterium is called **lysogenic** or **phage conversion**. In transduction, the phage acts only as a vehicle carrying bacterial genes from one cell to another but in lysogenic conversion the phage DNA itself is the new genetic element. Lysogenic conversion influences sensitivity to bacteriophages (immunity to superinfection with the same or related phages) and antigenic characteristics. Of great medical importance is the lysogenic conversion in diphtheria bacilli, which acquire Toxigenicity by lysogenization with the phage beta. Elimination of the phage from a toxigenic strain renders it nontoxigenic.

Significance: Lysogenic (phage) conversion occurs in nature and is the source of virulent strains (toxigenic) of bacteria.

Conjugation is transfer of DNA from a donor to a recipient by direct physical contact between the cells. In bacteria there are two mating types a donor (male) and a recipient (female) and the direction of transfer of genetic material is one way; DNA is transferred from a donor to a recipient.

Mating types in bacteria

The ability of a bacterium to be a **donor** is a consequence of the presence in the cell of an extra piece of DNA (**F factor** or **fertility factor** or **sex factor)**. The F-factor has genes on it that are needed for its replication and for its ability to transfer DNA to a recipient. One of the things the F-factor codes for is the ability to produce a **sex pilus** (**F-pilus**) on the surface of the bacterium. This pilus is important in the conjugation process (formation of *conjugative bridge* or tube). The ability to act as a r**ecipient** is a consequence of the lack of the F-factor.

Physiological states of the F-factor:

1. **Autonomous (F^+-cell):** In this state the F-factor carries only those genes necessary for its replication and for DNA transfer. There are no chromosomal genes associated with the F-factor in F^+ strains. In crossing of the type F^+ and F^- the F^- becomes F^+ while F^+ remains F^+. Thus, the F-factor is *infectious*. In addition, there is only *low* level transfer of chromosomal genes!

2. **Integrated (Hfr-cell)**: In this state the F-factor has integrated into the bacterial chromosome through a recombination event. In crossing of the type Hfr and F^- the F^-

rarely becomes Hfr and Hfr remains Hfr. In addition, there is a high frequency of transfer of donor chromosomal genes.

3. **Autonomous with chromosomal genes (F'-cell)**: In this state the F-factor is autonomous but it now carries some chromosomal genes. F'-factors are produced by excision of the F-factor from an Hfr. Occasionally, when the F-factor is excising from the Hfr chromosome, donor genes on either side of the F-factor can be excised with the F-factor generating an F'. F'-factors are named depending on the chromosomal genes that they carry. In crossing of the type F' and F^- the F^- becomes F' while F' remains F'. In addition there is *high* frequency of transfer of those chromosomal genes on the F' and *low* frequency transfer of other donor chromosomal genes.

Significance of conjugation: Among the Gram-negative bacteria this is the major way that bacterial genes are transferred. Transfer can occur between different species of bacteria. Transfer of multiple antibiotic resistance by conjugation has become a major problem in the treatment of certain bacterial diseases. Since the recipient cell becomes a donor after transfer of a plasmid it is easy to see why an antibiotic resistance gene carried on a plasmid can quickly convert a sensitive population of cells to a resistant one.

Gram-positive bacteria also have plasmids that carry multiple antibiotic resistance genes, in some cases these plasmids are transferred by conjugation while in others they are transferred by transduction. The mechanism of conjugation in Gram-positive bacteria is different than that for Gram-negative. In Gram-positive bacteria the donor makes an adhesive material which causes aggregation with the recipient and the DNA is transferred.

Genetic regulatory mechanisms

Bacteria do not make all the proteins that they are capable of making all of the time. Rather, they can *adapt* to their environment and make only those gene products that are essential for them to survive in a particular environment.

For example, bacteria do not synthesize the enzymes needed to make *tryptophan* when there is an abundant supply of tryptophan in the environment. However, when tryptophan is absent from the environment the enzymes are made. Similarly, just because a bacterium has a gene for resistance to an antibiotic does not mean that that gene will be expressed. The resistance gene may only be expressed when the antibiotic is present in the environment.

Bacteria usually control gene expression by regulating the *level of transcription*. In bacteria, genes with related function are generally located nearly to each other and they are regulated coordinately (*i.e.* when one is expressed they all are expressed). **Coordinate regulation** of clustered genes is accomplished by regulating the production of a *polycistronic mRNA* (*i.e.* a large mRNA containing the information for many genes). Thus, bacteria are able to «sense» their environment and express the appropriate set of genes needed for that environment by regulating transcription of those genes.

The operon model

Inducible genes are those in which the presence of a substance (an **inducer**) in the environment turns on the expression of one or more genes (structural genes) involved in the metabolism of that substance (lactose induces the expression of the lac-genes. An antibiotic induces the expression of a resistance gene). **Induction** is common in metabolic pathways that result in the catabolism of a substance and the inducer is normally the substrate for the pathway.

Lactose operon

The lactose operon contains *three structural genes* that code for enzymes involved in lactose metabolism (galactosidase, permease, galactose transacetylase). These genes are transcribed from a common promoter into a polycistronic mRNA, which is translated to get these three enzymes.

The expression of the structural genes is not only influenced by the presence or absence of the inducer, it is also controlled by a **specific regulatory gene**. The regulatory gene may be next to or far from the genes that are being regulated. The regulatory gene codes for a specific protein product called a **repressor**.

The repressor acts by binding to a specific region of the DNA called the **operator** which is adjoined to the structural genes being regulated. The structural genes together with the operator region and the promoter are called an operon (see figure 50).

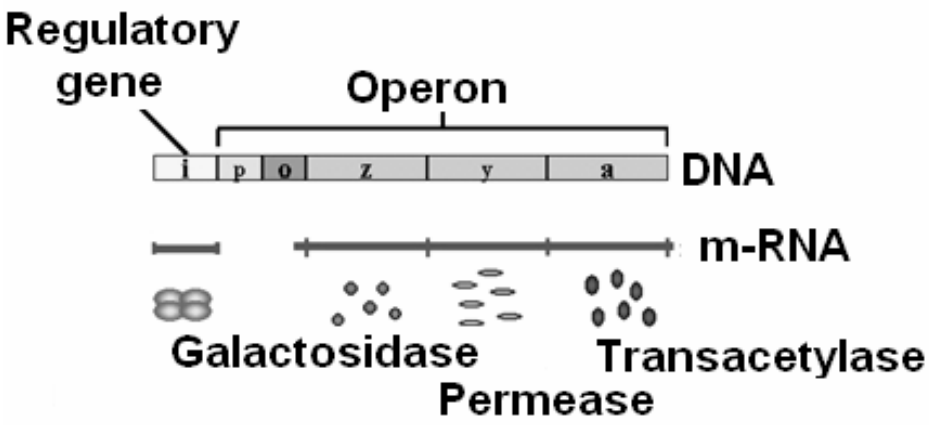


Figure 50 — Scheme of lactose operon

However, the binding of the repressor to the operator is prevented by the inducer and the inducer can also remove repressor that has already bound to the operator. Thus, in the presence of the inducer the repressor is inactive and does not bind to the operator, resulting in transcription of the structural genes.

In contrast, in the absence of inducer the repressor is active and binds to the operator, resulting in inhibition of transcription of the structural genes. This kind of control is referred to a **negative control** since the function of the regulatory gene product (repressor) is to turn off transcription of the structural genes (see figure 51).

Methods of genetics applied in microbiology

Discoveries in microbial genetics have provided the basis for the discipline of molecule genetics, which is concerned with the analysis and manipulation of DNA using biochemical and microbiological techniques. Some techniques and application of molecule genetics are discussed below:

1. **Molecular analysis** (content in percent of G+C nucleotides in bacterial genome).
2. **Genetic engineering**.
3. **DNA probe**.
4. **Blotting techniques**.
5. **Polymerase chain reaction** (PCR).
6. **Gene sequencing machines and techniques**

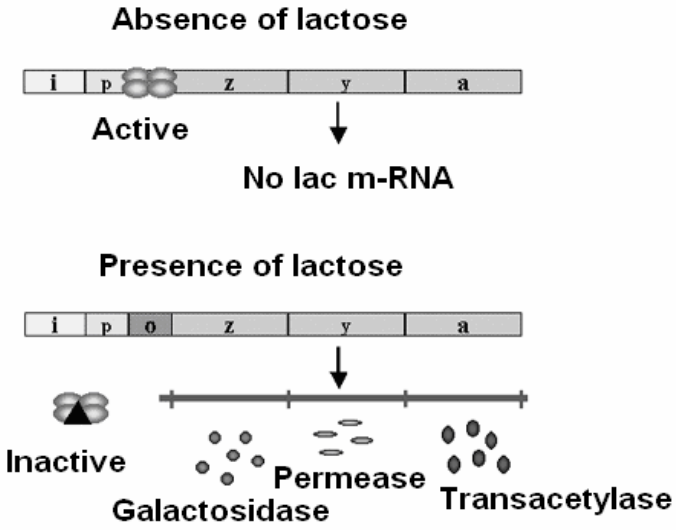


Figure 51 — Negative control of lactose operon

Molecular analysis

In the past several years, sequencing of the entire genomes of one representative (i.e. a strain) of a few bacterial species has been achieved. In each case, this has involved massive amounts of work by large research groups dedicated to the task of sequencing. Alternatively, genomic similarity has been historically assessed by the content of guanine (G) plus cytosine (C), usually expressed as a percentage (% GC).

DNA-DNA homology (or how well two strands of DNA from different bacteria bind [hybridize] together) is employed to compare the genetic relatedness of bacterial strains/species. If the DNA from two bacterial strains display a high degree of homology (i.e. they bind well), the strains are considered to be members of the same species. DNA from different bacterial species (unless closely related) display no homology.

In the last few years, sequencing of 16S ribosomal RNA molecules (16S rRNA) has become the "gold standard" in bacterial taxonomy. The molecule is approximately sixteen hundred nucleotides in length. The sequence of 16S rRNA provides a measure of genomic similarity above the level of the species allowing comparisons of relatedness across the entire bacterial kingdom. Closely related bacterial species often have identical rRNA sequences. The technique thus provides complementary information to DNA-DNA hybridization. Determinations of the sequence of 16S rRNA genes and other genetic regions are used in identification in the clinical microbiology laboratory.

Blotting techniques

DNA fragments obtained by restriction enzyme digestion and separation on gel can be transferred from the gel by **blotting** to nitrocellulose nylon membranes that bind the DNA. The DNA bound to the membrane is denatured and treated with *radioactive single-stranded DNA probes*. These will hybridise with homologous DNA to form *radioactive double-stranded segments*, which can be detected on X-ray film.

This highly sensitive technique for identifying DNA fragments by DNA. DNA hybridisation is called *Southern blotting,* after E. M. Southern who devised it. This technique has very wide application in DNA analysis.

An analogous procedure for the analysis of RNA has been called *Northern blotting* (as opposed to southern blotting!). Here the RNA mixture is separated by gel electrophoresis, blotted and identified using labelled DNA or RNA probes.

A similar technique for the identification of proteins (antigens) is called ***immunoblotting*** (or, in conformity with other blotting techniques, *western blotting).* Here the protein *antigen mixture* is separated by SDS-PAGE (sodium dodecyl sulfate-poly-acrylamide gel electrophoresis), blotted onto nitrocellulose strips and identified by *enzyme labelled antibodies* as probes.

The western blot test has received wide publicity as the **confirmatory test** for the diagnosis of **HIV** antibody in sera. The specificity of the test depends on its ability to separately identify antibodies directed against different antigens of the pathogen (for example, against the surface, core and reverse transcriptase antigens of HIV).

Genetic engineering

The most important application of molecule genetics in biotechnology is genetic engineering or recombinant DNA technology. This consists of isolation of the genes coding for any desired proteins from microorganisms or from other form of life and their introduction into suitable organism, in which the genes would be functional, directing the production of the *specific protein*. Such cloning of genes in microorganisms enables the ***preparation of the desired protein in pure form, in large quantities and at a reasonable cost***.

Different strategies have been employed for obtaining the desired genes. The *DNA can be cleaved* by specific enzymes called **restriction endonucleases** and the fragments containing the *desired genes isolated*. This does not work with DNA of higher organisms as they contain *introns*. In such cases, the concerned messenger RNA can be isolated from cells producing the desired protein. A DNA copy is made from the mRNA using the enzyme **reverse transcriptase**. The double-stranded DNA gene is then prepared using **DNA polymerase**. This is *incorporated* into suitable ***vectors*** or carriers, such as **plasmids** or **temperate bacteriophages**, for insertion into microorganisms. The microorganism commonly employed is *E. coli* K12, though many other bacteria and yeasts have also been used.

Genetic engineering has become an established branch of biotechnology with great scope for commercial exploitation. Cloned human *insulin, interferons*, *somatostatin, growth hormones* and many other biologicals have already been marketed. *Safer vaccines* can be produced by cloning the protective antigens of pathogens.

Restriction endonucleases (*restriction enzymes*) are microbial enzymes which cleave double-stranded DNA at specific oligonucleotide sequences. Many such enzymes which act at different nucleotide sequences have been recognised. The natural function of restriction enzymes in bacteria may be the *destruction of foreign DNA* that may enter the bacterial cell.

DNA probes

The specificity of the interaction in base pairing during DNA or RNA synthesis enables the production of specific DNA *probes.* These are radioactive labelled copies of cloned single-stranded DNA fragments, usually 20–25 nucleotides long and containing *unique nucleotide sequences* which can be used for the detection of *homologous* DNA by hybridisation.

Probes containing sequences unique to the microbe to be detected can be added to microbial cultures, body fluids, tissues or other materials suspected to contain the microbe. **The DNA probe hybridises with the complementary specific sequences on the microbe's DNA.**

The advantages of DNA probes for diagnosis are their high degree of specificity, ability to detect *minute quantities of complementary DNA* even in the presence of other microbes, and the capacity to recognise microbes that are either difficult or impossible to culture.

Polymerase chain reaction

This is a rapid automated method for the amplification of specific DNA sequences (or genes), invented and patented in 1985 by Saiki. PCR consists of several cycles of *sequential DNA replication* where the products of the first cycle become the template for the next cycle. It makes available abundant quantities of specific DNA sequences starting from sources containing minimal quantities of the same!

The technique is as follows: two oligonucleotide primers complementary to the lateral region of the DNA sequence to be amplified are incubated with the target DNA, nucleotides and DNA polymerase.

The reaction consists of these essential steps:

1. Denaturation of the sample DNA to single strand;
2. Annealing of sequence-specific oligonucleotide primers to the boundaries of the DNA segment;
3. Extension of the primers by DNA polymerase to form new double-stranded DNA across the segment by sequential addition of deoxynucleotides.

These three steps constitute one cycle of the reaction. These cycles are repeated several times (20–50 cycles), at the end of which hundreds of thousands of copies of the original target sequences are available (see process in figure 52).

PCR is an essential element in DNA fingerprinting and in the sequencing of genes and entire genomes. Basically, it's like a technique to photocopy pieces of DNA. In a matter of a few hours, a single DNA sequence can be amplified to millions of copies. PCR lets scientists work with samples containing even very small starting amounts of DNA. The technique makes use of the DNA repair enzyme polymerase. This enzyme, present in all living things, fixes breaks or mismatched nucleotides in the double-stranded DNA helix. These breaks or mismatches could cause genes to malfunction if left unfixed. PCR is useful for diagnosis of infectious, genetic or neoplastic diseases, in judicial investigations, in archeobiological studies of ancient specimens and in the examination of phylogenetic relationships in evolution.

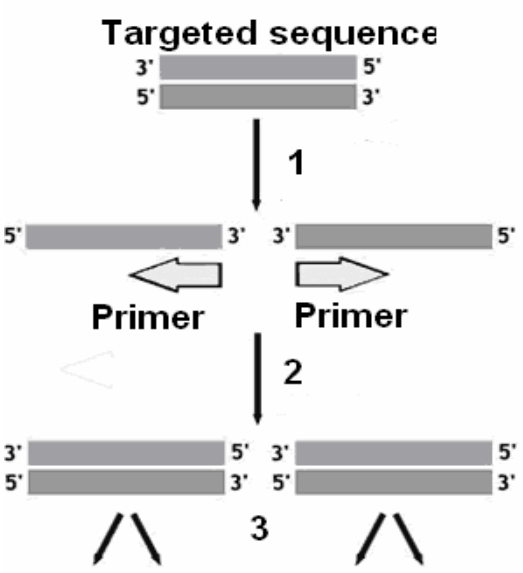


Figure 52 — PCR process

1. Heat, cool, add primers to get ssDNA that primers anneal to the boundaries of the DNA-segment. 2. To add polymerase and deoxynucleotides to synthesize complimentory strand. 3. To repeat cycle 20–50 times.

Gene sequencing machines and techniques

The advent of PCR has led to an explosion of microbial gene sequencing in recent years. PCR spells out the entire sequence of the nucleotide bases in a DNA molecule that code for a specific protein (figure 53). Scientists also use sequencing to spell out from start to end every single nucleotide in an organism's DNA — its entire genome.

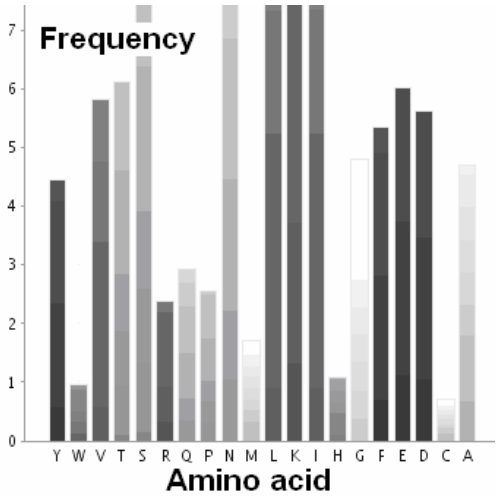


Figure 53 — Amino acids structure of *Mycoplasma penetrans*

Gene and genome sequencing involve a variety of computers, software programs, automated sequencing machines, fluorescent dyes, lasers, and other tools. The development of machines that can quickly chop up, separate, realign, and read bits of DNA have greatly speeded up the sequencing process. What used to take a person working by hand to do in a year can now be done by machines in just a few hours. Scientists use gene and genome sequences to precisely compare and differentiate organisms.

The role of microorganisms in genetic engineering

Bacteria in natural environments are continually exposed to a dilute "soup" of foreign DNA released from other organisms which have died or lysed. They are also exposed to bacteriophage DNA. If unable to protect themselves, they would rapidly become infected or killed. The answer to this problem is the many different *restriction-modification systems* possessed by different groups of bacteria. The enzymes are named after the organisms from which they were originally isolated.

The utility of **cloning** is partly analytical, i.e. it provides the ability to determine the genetic organization of particular regions or whole genomes (the human genome will soon be underway). However, it also facilitates the production of naturally-occurring, artificially-modified biological products by the expression of cloned genes. The ability to take a gene from one organism (e.g. man or a tree), clone it in *E. coli* and express it in another (e.g. yeast) is dependent on the universality of the genetic code, i.e. the triplets of bases which encode amino acids in proteins.

Biotechnology can be divided into 4 main areas:

1. Production of **biomass**
2. **Fermentations**: "life in the absence of oxygen". Today, the term is taken to mean: 'biological processes that occur in the dark & do not involve respiratory chains with oxygen or nitrate as electron acceptors'.
3. Harnessing of **microbial secondary metabolism**.
4. The use of **genetically manipulated organisms**.

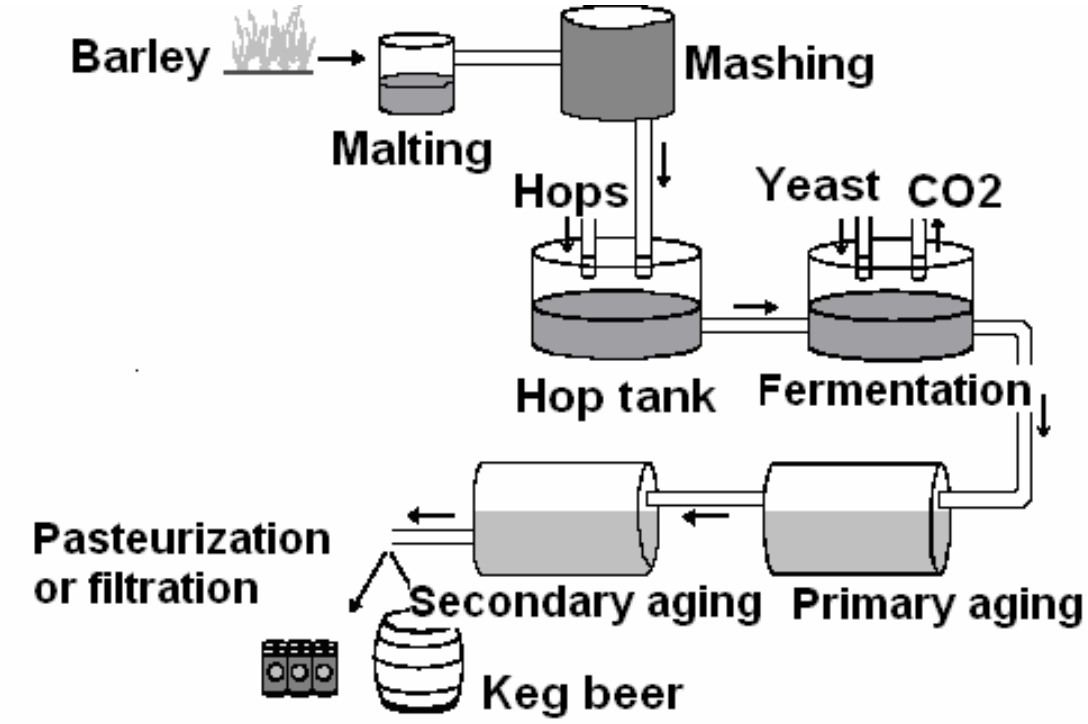


Figure 54 — Scheme of fermentation

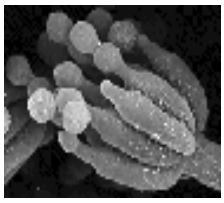
Fermentation (see figure 54):

- Biochemicals: aminoacids, citric acid, enzymes and vitamins.
- Transformations of steriods.
- Industrial chemicals.
- Foodstuffs: baking, dairy products and condiments.

Some of the products now being produced by genetic technology include:

- Vaccines.
- A number of human growth hormones, blood clotting agents, insulin & potential anti-cancer agents have been produced in bacteria.
- Insect control — two groups of microorganisms have been used as insecticides: *Bacillus thuringiensis* — produces a toxin which kills certain species of caterpillars.

The practical application of molecular biology is founded on our understanding of microorganisms. **Microbiology** is thus a central discipline in modern biology.



PATHOGENESIS OF BACTERIAL INFECTION

Infection and infection disease

Infection and immunity involve interaction between the animal body (host) and the infecting microorganism. Based on their relationship to their hosts, microorganisms can be classified as saprophytes and parasites. **Saprophytes** are free-living microbes that subsist on dead or decaying organic matter. They are found in soil and water and play an important role in the degradation of organic materials in nature. They are generally incapable of multiplying on living tissues and therefore are of little relevance in infectious disease. Exceptionally, however, some saprophytes like *B. subtilis* may infect devitalized hosts whose natural resistance is greatly reduced (*opportunistic infection*). **Parasites** are microbes that can establish themselves and multiply in hosts. Parasitic microbes may be *pathogens* (from Greek that is, disease-producing) or *commensals* (from Latin i.e., living together). **Pathogens** are microorganisms that are capable of producing disease in the host and their presence is abnormal; examples include *Mycobacterium tuberculosis* (tuberculosis) and *Yersinia pestis* (plague). **Commensal microbes** live in complete harmony with the host without causing any damage to it. The normal bacterial flora of the body consist largely of commensals. Many commensals behave as **facultative pathogens** (*opportunistic pathogens*) in that they can produce disease when the host resistance is lowered (immunosuppressed and debilitated persons). For example, *E. coli* is part of gastrointestinal flora in normal humans but is also is a common cause of urinary tract infections, traveler's diarrhea, and other diseases.

It is necessary to distinguish between the term **"infection"** and **"infectious disease"**. The lodgement and multiplication of a parasite in or on the tissues of a host constitute infection. It does not invariably result in disease! In fact, **disease** is a **rare consequence** of **infection,** which is a **common natural event. Infection** may imply colonization, multiplication, invasion or persistence of a pathogen on or within a host, but **infectious disease** is used to describe an infection that causes significant overt damage to the host.

Sources of infection

1. Human beings: The commonest source of infection for human beings are human beings themselves. The parasite may originate from a patient or a carrier. A **carrier** is a person who harbours the pathogenic microorganism without suffering from any ill-effect because of it. Several types of carriers have been identified. A **healthy carrier** is one who harbours the pathogen but has never suffered from the disease caused by the pathogen, while a **convalescent carrier** is one who has recovered from the disease and continues to harbour the pathogen in his body. Depending on the duration of carriage, carriers are classified as *temporary* and *chronic.* The **temporary carrier** state lasts less than six months, while **chronic carriage** may last for several years and sometimes even for the rest of one's life. The term **contact carrier** is applied to a person who acquires the pathogen from a patient, while the term **paradoxical carrier** refers to a carrier who acquires the pathogen from another carrier.

2. Animals*:* Many pathogens are able to infect both human beings and animals. Animals may, therefore, act as sources of human infection. In some instances, the infection in animals may be asymptomatic. Such animals serve to maintain the parasite in nature and act as the *reservoir* of human infections. Infectious diseases transmitted from animals to human beings are called ***zoonoses****.* Zoonotic diseases may be bacterial (plague from rats), viral (rabies from dogs), protozoal (toxoplasmosis from cats), helminthic (hydatid disease from dogs) or fungal (zoophilic dermatophytes from cats and dogs).

3. Insects: Blood sucking insects may transmit pathogens to human beings. The diseases so caused are called ***arthropod borne diseases****.* Insects such as mosquitoes, ticks, mites, flies, fleas and lice that transmit infections are called ***vectors****.* Transmission may be mechanical (for example, transmission of dysentery or typhoid bacilli by the domestic fly). Such vectors are called *mechanical vectors.* In other instances, the pathogen multiplies in the body of the vector, often undergoing part of its developmental cycle in it. Such vectors are termed *biological vectors* (for example, Anopheli mosquito in malaria).

4. Soil and water: Some pathogens can survive in the soil for very long periods. Spores of tetanus bacilli may remain viable in the soil for several decades and serve as the source of infection. Water may act as the source of infection due to contamination with pathogenic microorganisms (cholera vibrio, infective hepatitis virus) or due to the presence of aquatic vectors (cyclops in guinea worm infection).

5. Food: Contaminated food may act as a source of infection. The presence of pathogens in food may b due to external contamination (food poisoning by staphylococcus) or due to preexistent infection in meat or other animal products (salmonellosis).

Methods of transmission of infection

Contact: Infection may be acquired by contact, which may be direct or indirect. Sexually transmitted diseases such as syphilis and gonorrhea illustrate spread by direct contact. Indirect contact may be through the agency inanimate object; such as clothing, pencils or toys which may be contaminated by a pathogen from one person and act as a vehicle for its transmission to another.

Inhalation *(airborne mechanism)*: Respiratory infections such as influenza and tuberculosis are transmitted by inhalation of the pathogen. Such microbes are shed by the patients into the environment, in secretions from the nose or throat during sneezing, speaking or coughing. Large drops of such secretions fall to the ground and dry there. Pathogens resistant to drying may remain viable in the dust and act as sources of infection.

Ingestion *(faecalo-oral mechanism)***:** Intestinal infections are generally acquired by the ingestion of food or drink contaminated by pathogens. Infection transmitted by ingestion may be waterborne (cholera), foodborne (food poisoning) or handborne (dysentery).

Inoculation: Pathogens, in some instances, may be inoculated directly into the tissues of the host. Tetanus spores implanted in deep wounds, rabies virus deposited subcutaneously by dog bite and arboviruses injected by insect vectors are examples. Infection by inoculation may be **iatrogenic** when unsterile syringes and surgical equipment are employed. Hepatitis B and the Human Immunodeficiency Virus (HIV) may be transmitted through transfusion of infected blood, or the use of contaminated syringes and needles, particularly among addicts of injectable drugs.

Insects *(transmissive mechanism)***:** Insects may act as mechanical or biological vectors of infectious diseases.

Congenital: Some pathogens are able to cross the placental barrier and infect the fetus in utero. This is known as *vertical transmission.* This may result in abortion, miscarriage or stillbirth. Live infants may be born with manifestations of a disease, as in congenital syphilis. Intrauterine infection with the rubella virus, especially in the first trimester of pregnancy, may interfere with organogenesis and lead to congenital malformation. Such infections are known as *teratogenic* infections.

Iatrogenic *and laboratory* *infections:* Infection may sometimes be transmitted during administration of injections, lumbar puncture and catheterization, if care in asepsis is lacking. Modern methods of treatment such as exchange transfusion, dialysis, and heart and transplant surgery have increased the possibilities for iatrogenic infections. Laboratory personnel handling infectious material are at risk and special care should be taken to prevent laboratory infection.

The outcome of an infection will depend on the interaction between microbial factors which predispose to pathogenicity and host factors which contribute to resistance!

Classification of infections

Infections may be classified in various ways. Initial infection with a parasite in a host is termed **primary infection***.* Subsequent infections by the same parasite in the host are termed **reinfections***.* When a new parasite sets up an infection in a host whose resistance is lowered by a preexisting infectious disease, this is termed **secondary infection***.*

When in a patient already suffering from a disease a new infection is set up from another host or another external source, it is termed **cross infection***.* Cross infections occurring in hospitals are called *nosocomial infections* (from Greek, *nosocomion* hospital). The term *eiatrognic infection* refers to physician induced infections resulting from investigative, therapeutic or other procedures.

Depending on whether the source of infection is from the host's own body or from external sources, infections are classified as *endogenous* or *exogenous,* respectively. **Autoinfection** is endogenic infection is caused by own potential-pathogenic microflora.

Based on the clinical effects of infections, they may be classified into different varieties. **Inapparent infection** is one where clinical effects are not apparent. The term **subclinical infection** is often used as a synonym.

Atypical infection is one in which the typical or characteristic clinical manifestations of the particular infectious disease are not present. Some parasites, following infection, may remain in the tissues in a latent or hidden form proliferating and producing clinical disease when the host resistance is lowered. This is termed **latent infection.**

Types of infectious diseases

The periods of development of the infectious disease:

1. Incubatory period (time between pathogenic invasion in macroorganism and display of the clinical symptoms).
2. Predromal period (display of the first nonspecific symptoms: weakness, head pain, increase of the body T^0).
3. Period of clinical displays of infectious disease (maximal display of the clinical symptoms of given disease).
4. Outcome of infectious disease (reconvalescence, microbocarriage, transition in the chronic stage, death).

Infectious diseases may be **localised** or **generalized**. Localised infections may be superficial or deep-seated. Generalized infection involves the spread of the infecting agent from the site of entry by contiguity, through tissue spaces or channels, along the lymphatics or through the bloodstream. Circulation of bacteria in the blood is known as ***bacteremia (sepsis)***. Transient bacteremia is a frequent event even in healthy individuals and may occur during chewing, brushing of teeth or straining at stools. The bacteria are immediately mopped up by phagocytic cells and are unable to initiate infection. Bacteremia of greater severity and longer duration is seen during generalized infections as in typhoid fever. ***Septicemia*** is the condition where bacteria circulate and multiply in the blood, form toxic products and cause high, swinging type of fever. ***Pyemia*** is a condition where pyogenic bacteria produce septicemia with multiple abscesses in the internal organs such as the spleen, liver and kidney. **Virusemia** is a condition where viruses circulate in the blood. **Toxemia** is a condition where endotoxins in the blood. **Toxenemia** is a condition where exotoxins in the blood.

Depending on their spread in the community, infectious diseases may be classified into different types. ***Endemic*** diseases are those which are constantly present in a particular area. Typhoid fever is endemic in most parts of India. An ***epidemic*** disease is one that spreads rapidly, involving many persons in an area at the same time. Influenza causes annual winter epidemics in the cold countries. *A.* ***pandemic*** is an epidemic that spreads through many areas of the world involving very large numbers of people within a short period. Influenza, cholera, plague and enteroviral conjunctivitis are pandemic diseases. Epidemics vary in the rapidity of spread. Waterborne diseases such as cholera and hepatitis may cause explosive outbreaks, while diseases which spread by person-to-person contact evolve more slowly.

Factors predisposing to microbial pathogenicity

The terms “pathogenicity” and “virulence” refer to the ability of a microbe to produce disease or tissue injury but it is convenient to make a fine distinction between them. “**Pathogenicity**” is generally employed to refer to the ability of a microbial *species* to produce disease, while the term “**virulence**” is applied to the same property in a *strain* of microorganism. Thus the species *M. tuberculosis* is referred to as being pathogenic. The pathogenic species *M. tuberculosis* contain strains of varying degrees of virulence including those which are avirulent, such as the vaccine strains. The virulence of a strain is not constant and may undergo spontaneous or induced variation. Enhancement of virulence is known as *exaltation*. Reduction of virulence is known as *attenuation* and can be achieved by passage through unfavourable hosts, repeated cultures in artificial media, growth under high temperature or in the presence of weak antiseptics, desiccation, or prolonged storage in culture.

So, microbes express their pathogenicity by means of their **virulence**, a term which refers to the **degree of pathogenicity** of the microbe. Hence the **determinants of virulence** of a pathogen are any of its genetic or biochemical or structural features that enable it to produce disease in a host. The relationship between a host and a pathogen is dynamic, since each modifies the activities and functions of the other. The outcome of an infection depends on the **virulence of the pathogen** and the relative degree of resistance or susceptibility of the host, due mainly to the effectiveness of the host defense mechanisms. Microbial **pathogenicity** has been defined as the structural and biochemical mechanisms whereby microorganisms cause disease. Pathogenicity in bacteria may be associated with unique structural components of the cells (e.g. capsules, fimbriae, LPS or other cell wall components) or active secretion of substances that either damage host tissues or protect the bacteria against host defenses.

There are two broad qualities of pathogenic bacteria underlie the means by which they cause disease:

1. The ability to invade tissues**: Invasiveness**, which encompasses mechanisms for colonization (adherence and initial multiplication), ability to bypass or overcome host defense mechanisms, and the production of extracellular substances ("invasions") which facilitate invasion**.**

2. The ability to produce toxins: **Toxigenesis.** Toxic substances, both soluble and cell-associated, may be transported by blood and lymph and cause cytotoxic effects at tissue sites remote from the original point of invasion or growth. Bacteria produce two types of toxins called exotoxins and endotoxins.

Factors of microbial pathogenicity

● The initial event in the pathogenesis of many infections is the **attachment** of the bacteria to body surfaces. This attachment is not a chance event but a specific reaction between surface *receptors* on host cells and adhesive structures *(ligands)* on the surface of bacteria. These adhesive structures are called ***adhesins***.

Adhesins may occur as organized structures, such as *fimbriae* and *pili*, or as *colonisation factors*. This specific adhesin may account for the **tissue tropisms** and host specificity exhibited by many pathogens. Adhesins serve as virulence factors, and loss of adhesins often renders the strain avirulent. Adhesins are usually made of protein and are antigenic in nature.

Colonization: the establishment of the pathogen at the appropriate portal of entry. Pathogens usually colonize host tissues that are in contact with the external environment. Sites of entry in human hosts include the urogenital tract, the digestive tract, the respiratory tract and the conjunctiva. Organisms that infect these regions have usually developed tissue adherence mechanisms and some ability to overcome or withstand the constant pressure of the host defenses on the surface.

Table 14 is a list of terms that are used in medical microbiology to refer to microbial adherence to surfaces or tissues.

● **Invasiveness:** This refers to the ability of a pathogen to spread in the host tissues after establishing infection.

Highly invasive pathogens characteristically produce spreading or generalized lesions (e.g., streptococcal septicemia following wound infection), while less invasive pathogens cause more localised lesions (e.g., staphylococcal abscess). Some pathogens though capable of causing serious or even fatal diseases lack invasiveness altogether (e.g., the tetanus bacillus which remains confined to the site of entry and produces the disease by producing a potent toxin).

Invasion of a host by a pathogen may be aided by the production of bacterial extracellular substances which act against the host by breaking down primary or secondary defenses of the body. Medical microbiologists have long referred to these substances as **invasions** are proteins (enzymes) that act locally to damage host cells and/or have the immediate effect of facilitating the growth and spread of the pathogen. The damage to the host as a result of this invasive activity may become part of the pathology of an infectious disease.

Heterotrophic bacteria, in general, produce a wide variety of **extracellular enzymes** including proteases, lipases, glycohydrolases, nucleases, etc., which are not clearly shown to have a direct role in invasion or pathogenesis. These enzymes presumably have other functions related to bacterial nutrition or metabolism, but may aid in invasion either directly or indirectly.

Table 14 — Factors of adherence and colonization

Aadherence factor	Description
Adhesin	Surface structure or macromolecule that binds a bacterium to a specific surface
Receptor	Complementary macromolecular binding site on a (eukaryotic) surface that binds specific adhesins
Fimbriae	Filamentous proteins on the surface of bacterial cells that may behave as adhesins for specific adherence
Glycocalyx	Layer of polysaccharide fibers on the surface of bacterial cells which may be involved in adherence to a surface
Capsule	Detectable layer of polysaccharide (rarely polypeptide) on the surface of a bacterial cell which may mediate specific or nonspecific attachment
LPS	Distinct cell wall component of the outer membrane of Gram-negative bacteria with the potential structural diversity to mediate specific adherence. Probably functions as an adhesin
Teichoic acids	Cell wall components of Gram-positive bacteria that may be involved in nonspecific or specific adherence

● **Toxigenicity and toxicity:** Bacteria produce two types of toxins — exotoxins and endotoxins. ***Exotoxins*** (products of toxigenicity) are heat labile proteins which are secreted by certain species of bacteria and diffuse readily into the surrounding medium. They are highly potent in minute amounts and constitute some of the most poisonous substances known. One mg of tetanus or botulinum toxin is sufficient to kill more than one million guinea pigs and it has been estimated that 3 kg of botulinum toxin can kill all the inhabitants of the world!

Table 15 — Characteristics of endotoxin and exotoxin

Property	Exotoxin	Endotoxin
1. **Bacterial source**	Gram-positive and gram-negative bacteria	Only gram-negative bacteria
2. **Location**	Actively secreted by cells; diffuse into surrounding medium	Form part of the cell wall; do not diffuse into surrounding medium
3.**Composition**	Protein	LPS (lipid A)
4. **Stability**	Heat labile (denatured by boiling)	Heat stable, not destroyed by autoclaving
5. **Action**	Specific tissue affinities (usually bind to specific receptors on cells)	No specific tissue affinities (specific receptors do not find on cells)
6. **Antigenicity**	Highly antigenic; stimulate formation of high-titer antitoxin	Weakly antigenic. Relationship between Ab-titers and protection from disease is less clear than with endotoxins.
7. **Specificity**	Specific effect for each exotoxin	Effect nonspecific (common action to all endotoxins)
8. **Enzymatic activity**	Action often enzymatic	No enzymatic action
9. **Pyrogenicity**	Usually do not produce fever in the host	Usually produce fever in the host by release of interleukin-1 and other mediators
10. **Effective dose**	Active in very minute doses	Active only in very large doses
11. **Interaction with specific antibody**	Action specifically neutralized by antibody	Neutralization by antibody is ineffective
12. **Obtaining**	Readily separable by physical means such as filtration	Obtained only by cell lysis

Inactivated exotoxins by formaldehyde are called **toxoids** which are nontoxic but retain the ability to induce antibodies (**antitoxins**). They exhibit specific tissue affinities and pharmacological activities, each toxin producing a typical effect which can be made out by characteristic clinical manifestations. Exotoxins are generally formed by Gram positive bacteria but may also be produced by some Gram negative organisms such as *Shiga's dysentery bacillus*, *cholera vibrio* and enterotoxigenic *E. coli.*

Endotoxins (products of toxicity) are heat stable lipopolysaccharides (LPS) which form an integral part of the cell wall of Gram negative bacteria. Their toxicity depends on the lipid component (lipid A). They are not secreted outside the bacterial cell and are released only by the disintegration of the cell wall. They cannot be toxoided. They are poor antigens and their toxicity is not completely neutralized by the homologous antibodies. They are active only in relatively large doses. They do not exhibit specific pharmacological activities. All endotoxins, whether isolated from pathogenic or nonpathogenic bacteria, produce similar effects. Administration of small quantities of endotoxin in susceptible animals causes an elevation of body temperature manifested within 15 minutes and lasting for several hours.

The pyrogenic effect of fluids used for intravenous administration is usually due to the presence of endotoxins from contaminant bacteria. Intravenous injections of large doses of endotoxin and massive Gram negative septicemias cause **endotoxic shock** marked by fever, leucopenia, thrombocytopenia, significant fall in blood pressure, circulatory collapse and bloody diarrhea leading to death. LPS is among the different agents that can activate the alternative pathway of the complement cascade, accelerating a variety of complement-mediated reactions (anaphylatoxins, chemotactic responses, membrane damage, etc).

Table 16 — Classification of the bacterial exotoxins

Type	Group	Producer
1. Cytotoxin (inhibiting of protein synthesis)	Antielongaters	*Corynebacterium diphtheriae, Shigella flexneri, Shigella sonnei*
	Enterotoxins	*Staphylococcus aureus, Clostridium perfringens*
	Dermonecrotoxins	*Streptococcus pyogenes, Bordetella pertussis, Bacillus anthracis*
2.Membranetoxins (increasing of membrane permeability in result of membrane damage)	Leucocidins	*Staphylococcus aureus, Streptococcus pyogenes, Clostridium perfringens, Clostridium botulinum*
	Hemolysins	*Staphylococcus aureus, Clostridium perfringens*
	O-streptolysin	*Streptococcus pyogenes*
	Pneomalysin	*Streptococcus pneumoniae*
	α-toxin	*Clostridium perfringens*
	Tetanolysin	*Clostridium tetani*
3. Functional blockers (blocking of the functions of fabric system)	Thermostable enterotoxins	*Klebsiella pneumoniae, Escherichia coli*
	Thermolabile enterotoxins	*Escherichia coli, Salmonella Typhi*
	Choleragen	*Vibrio cholerae*
	Toxicoblocker "toxins" of the mouse	*Yersinia pestis, Bacillus anthracis*
	Neurotoxin	*Clostridium tetani, Clostridium botulinum*
4. Exfoliatins and erythrogenins (influence on the process of cell interaction)	Exfoliatins	*Staphylococcus aureus*
	Erythrogenins	*Streptococcus pyogenes*

Relative the exotoxin with a bacterial cell:

1. Completely secretable in environment (host) exotoxins (*C. diphtheriae, Cl.perfringens, St.aureus* (scarlet fever), *Str.pneumoniae*, pathogens of the anaerobic infections);

2. In part secretable in environment (host) exotoxins (*Cl.tetani Cl.botulinum, Sh.dysenteriae*);

3. Nonsecretable strongly connected with microbe toxins (*Yersinia pestis, E.coli).*

Genetic control of synthesis of the pathogenic factors:

1. Bacterial chromosome genes (formation of common pili, capsule of pneumococci, enzymes of invasion);

2. Plasmid genes (*Ent-plasmids* provide the synthesis of enterotoxins in enterotoxigenic strains of E.coli; *Hly-plasmids* provide the synthesis of hemolysin in St.aureus);

3. Genes introduced by temperate bacteriophages (corynephages provides by tox-genes of C. diphtheriae).

4. Plasmids: Genes coding for some virulence characteristics may be plasmid borne. Examples of plasmid borne virulence factors are surface antigens responsible for the colonisation of intestinal mucosa by *E. coli* and enterotoxin production by *E. coli* and *Staph, aureus.* Multiple drug resistance (R) plasmids increase the severity of clinical disease by their resistance to antibiotic therapy. **Bacteriophages***:* The classical example of phage directed virulence is seen in diphtheria. In diphtheria bacilli, the gene for toxin production is present in beta or other tox^+ corynephages.

5. Other bacterial products: Some bacterial products other than toxins, though devoid of intrinsic toxicity, may contribute to virulence by inhibiting the mechanisms of host resistance. *Pathogenic staphylococci* produce a thrombin-like **enzyme** *coagulase* which prevents phagocytosis by forming a fibrin barrier around the bacteria and walling off the lesion. *Fibrinolysins* promote the spread of infections by breaking down the fibrin barrier in tissues. *Hyaluronidases* split hyaluronic acid which is a component of intercellular connective tissue and thus facilitate the spread of infection along tissue spaces. *Leucocidins* damage polymorphonuclear leucocytes. Many pathogens produce hemolysins capable of destroying erythrocytes but their significance in pathogenicity is not clearly understood.

6. **Bacterial appendages:** Capsulated bacteria such as *pneumococci*, *K. pneumoniae* and *H. influenzae* are not readily phagocytosed. Some bacterial surface antigens such as the Vi-antigen of S. *Typhi,* K-antigens of *E. coli* also help the bacteria to withstand phagocytosis and the lytic activity of complement.

7. **Infecting dose (ID)***:* Successful infections require that an adequate number of bacteria should gain entry into the host. The dosage may be estimated as the *minimum infecting dose* **(MID)** or *minimum lethal dose* **(MLD)** which are, respectively, the minimum number of bacteria required to produce clinical evidence of infection or death, respectively, in a susceptible animal under standard conditions. **ID 50** and **LD 50**, as the dose required to infect or kill 50 per cent of the animals tested under standard conditions.

8. Route of infection: Some bacteria, such as streptococci, can initiate infection whatever be the mode of entry. Others can survive and multiply only when introduced by the optimal routes. Cholera vibrios are infective orally but are unable to cause infection when introduced subcutaneously (hypodermically). Bacteria also differ in their sites of election in the host body after introduction into tissues. They also differ in the ability to produce damage of different organs in different species of animals. The reasons for such selective multiplication in tissues are largely obscure, though they may be related to the presence in tissues of substances that may selectively hinder or favour their multiplication.

The most frequent portals of entry of pathogenic bacteria into the body are the sites where mucous meet with the skin: respiratory (upper and lower airways), gastrointestinal (primarily mouth), genital, and urinary tracts. Abnormal areas of mucous membranes and skin (eg, cuts, burns and other injuries) are also frequent sites of entry. Normal and skin provide the primary defense against infection. To cause disease, pathogens must overcome these barriers.

Ecology of the microorganisms

The normal microbial flora is more or less constant for each species and is broadly divided into residents and transients. The normal microbial flora plays an important role in body economy. They can 1) become pathogenic when host defences go down, 2) prevent or interfere with colonisation/invasion of the body by pathogens, 3) raise the immune status of the host against pathogens having related or shared antigens, and 4) cause confusion in diagnosis due to their ubiquitous presence in the body and their resemblance to some of the pathogens. Members of the normal flora form part of the host and include saprophytes, commensals, facultative pathogens and true pathogens.

The **microflora of the intestinal tract** synthesise vitamin K and several B vitamins which supply on occasion the body's needs. The antibiotic substances produced by some, for example, **colicins**, have a harmful effect on pathogens. The endotoxins liberated by them may help the defence mechanism of the body by triggering the *alternative complement pathway*, as long as they are not produced in excessive amounts.

On the contrary, the opportunistic pathogens among them cause disease when the body's defence mechanisms fail. Their abnormal multiplication can cause diseases such as *enteritis* and *endotoxic shock*.

Normal flora of the skin

The human skin is constantly and continuously bombarded by organisms present in the environment. It is also contaminated by one's own secretions and excretions, the extent depending on the individual's personal hygiene. The flora depends on the area of the body, the clothing one wears, one's occupation and environment.

Cultures from the skin have frequently demonstrated diphtheroids (including propionibacteria); staphylococci (aerobic and anaerobic); Gram positive aerobic spore bearing bacilli; *Str. viridans; Str.faecalis;* Gram negative bacilli such as *E. coli,* Proteus, and other intestinal organisms; mycobacteria (nonpathogenic); *Candida albicans;* cryptococci.

Often the skin of the face, neck, hands and buttocks carries pathogenic hemolytic streptococci and staphylococci. Penicillin resistant staphylococci are seen in individuals working in hospitals.

Normal flora of the conjunctiva, nose and nasopharynx

The conjunctiva is relatively free from organisms due to the flushing action of tears. The predominant organisms of the eye are diphtheroids *(Corynebacterium xerosis),* Moraxella species, staphylococci and nonhemolytic streptococci.

The floor of the nose harbours corynebacteria, staphylococci and streptococci. *Haemophilus* species and *Moraxella lacunata* may also be seen.

The nasopharynx of the infant is sterile at birth but, within 2–3 days after birth, acquires the common commensal flora and the pathogenic flora carried by the mother and the attendants. The nasopharynx can be considered the natural habitat of the common pathogenic bacteria which cause infections of the nose, throat, bronchi and lungs. Certain Gram negative organisms from the intestinal tract such as *Psedomonas aeruginosa, E.coli,* Proteus are also occasionally found in normal persons.

Normal flora of the mouth and upper respiratory tract

The mouth contains abundance of organisms — pigmented and nonpigmented micrococci, some of which are aerobic, Gram positive aerobic spore bearing bacilli, coliforms, Proteus and lactobacilli. The gum pockets between the teeth and the crypts of the tonsils have a wide spectrum of anaerobic flora — anaerobic micrococci, microaerophilic and anaerobic streptococci, vibrios, fusiform bacilli, corynebacterium species, actinomyces, mycoplasma, neisseria, and bacteroides are all found in varying extents.

The mouth of the infant is not sterile at birth. It generally contains the same types of organisms in about the same relative numbers as those present in the mother's vagina that is a mixture of micrococci, streptococci, coliform bacilli and lactobacilli. These organisms diminish in number during the first 2–5 days after birth and are replaced by the types of bacteria present in the mouth of the mother and nurse.

Within 12 hours after birth alpha hemolytic streptococci are found in the upper respiratory tract and become the dominant organisms of the oropharynx and remain so for life. In the pharynx and trachea, flora similar to that of the mouth establish themselves. Few bacteria are found in normal bronchi. Smaller bronchi and alveoli are normally sterile.

Normal flora of the intestinal tract

In breast fed children the intestine contains lactobacilli (L. *bifidus* constituting 99 per cent of total organisms in the feces), enterococci, colon bacilli and staphylococci. In artificilly fed (bottle fed) children *L. acidophilus* and colon bacilli and in part by enterococci, Gram positive aerobic and anaerobic bacilli. With the change of food to the adult pattern, the flora change. Diet has a marked influence on the relative composition of the intestinal and fecal flora.

The number of bacteria increases progressively beyond the duodenum to the colon, being comparatively low in the small intestine. In the adult duodenum there are 10^3–10^6 bacteria per gram, in the jejunum and proximal ileum 10^5–10^8 bacteria per gram of contents. In the duodenum and upper ileum, lactobacilli and enterococci predominate but in the lower ileum and cecum the flora resemble the fecal flora. There are about 10^{11} bacteria per gram of contents in the colon and rectum, constituting 10–20 per cent of the fecal mass. In the adult normal colon, the resident bacterial flora are mostly (96–99 per cent) anaerobes — anaerobic streptococci, anaerobic lactobacilli, clostridia, and bacteroides and about 1–4 per cent aerobes — enterococci, conforms, and small numbers of Proteus, Pseudomonas, lactobacilli, mycoplasma, Candida and others.

Normal flora of the urogenital tract

Mycobacterium smegmatis, a harmless commensal, is found in the genitalia of both men and women. From apparently normal men, aerobic and anaerobic bacteria can be cultured from a high proportion, including lactobacilli, *Gard. vaginalis, alpha hemolytic streptococci* and *Bacteroids species. Chlam. trachomatis* and *Ureaplasma urealyticum* may also be present. The female urethra is either sterile or contains a few Gram positive cocci.

The vulva of the newborn child is sterile but after 24 hours it acquires a varied flora of nonpathogenic organisms from the skin, vagina and intestines. The nature of the flora in the vagina depends on the pH of its secretions and its enzyme content. At birth the vagina is sterile. In the first 24 hours it is invaded by micrococci, enterococci and diphtheroids. In 2–3 days, the maternal estrin induces glycogen deposition in the vaginal epithelium. This facilitates the growth of lactobacilli which

produces acid from glycogen, and the flora for a few weeks is similar to that of the adult. During pregnancy there is an increase in *Staphylococcus epidermidis* and yeasts. Occasionally other members of the intestinal flora may be present. The normal vaginal flora often includes anaerobic cocci and bacilli, listeria, anaerobic streptococci, mycoplasma, *Gardnerella vaginalis,* neisseriae and spirochetes.

Bacteria in the blood and tissues

The commensals from the normal flora of the mouth, nasopharynx and intestinal tract may get into the blood and tissues. They are usually quickly eliminated by the normal defence mechanisms of the body. Occasional isolation of diphtheroids or nonhemolytic streptococci from normal and abnormal lymph nodes may be those which escaped elimination. Unless the organisms of doubtful pathogenicity are isolated more than once in serial blood cultures, they have little significance.

The benefits of the normal flora

The indigenous bacteria of the gastrointestinal tract of an animal, perhaps mainly as a consequence of their great numbers, seem to have the greatest overall impact on their host. The nature of the interactions between an animal host and its normal flora has been inferred from the study of **germ-free animals** (animals which lack any bacterial flora) compared to **conventional animals** (animals which have a typical normal flora). Following are the primary beneficial effects of the normal flora that are derived from these studies.

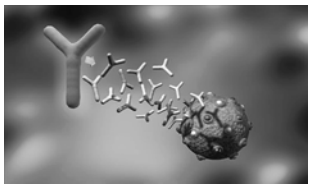
1. The normal flora synthesizes and excretes vitamins in excess of their own needs, which can be absorbed as nutrients by the host. For example, enteric bacteria secrete Vitamin K and Vitamin B12, and lactic acid bacteria produce certain B-vitamins. Germ-free animals may be deficient in Vitamin K to the extent that it is necessary to supplement their diets.

2. The normal flora prevents colonization by pathogens by competing for attachment sites or for essential nutrients. This is thought to be their most important beneficial effect, which has been demonstrated in the oral cavity, the intestine, the skin, and the vaginal epithelium. In some experiments, germ-free animals can be infected by *Salmonella* bacteria, while the infectious dose for conventional animals is near 10^6 cells.

3. The normal flora may antagonize other bacteria through the production of substances which inhibit or kill nonindigenous species. The intestinal bacteria produce a variety of substances ranging from relatively nonspecific fatty acids and peroxides to highly specific bacteriocins, which inhibit or kill other bacteria.

4. The normal flora stimulate the development of certain tissues, i.e., the caecum and certain lymphatic tissues (Peyer's patches) in the GI tract. The caecum of germ-free animals is enlarged, thin-walled, and fluid-filled, compared to that organ in conventional animals. Also, based on the ability to undergo immunological stimulation, the intestinal lymphatic tissues of germ-free animals are poorly-developed compared to conventional animals.

5. The normal flora stimulates the production of cross-reactive antibodies. Since the normal flora behave as antigens in an animal, they induce an immunological response, in particular, an antibody-mediated immune (AMI) response. Low levels of antibodies produced against components of the normal flora are known to cross react with certain related pathogens, and thereby prevent infection or invasion. Antibodies produced against antigenic components of the normal flora are sometimes referred to as "natural" antibodies, and such antibodies are lacking in germ-free animals.



GENERAL IMMUNOLOGY

Immunology is a science that studies immunity. Historically, immunity has been understood as a defense against, or resistance to, contagious (infectious) diseases. It has become apparent, however, that the mechanisms that confer protection against the above diseases also operate when a body mounts a reaction against some innocuous substances. Such a reaction is triggered when certain substances that are not made in the body ("foreign" substances) invade the body from outside. The mechanisms of immunity can protect against diseases that might be caused by the foreign agents but, on the other hand, these same mechanisms can themselves injure the body and cause disease. This mechanism is quite complex, involves many different cells, molecules, and genes (collectively termed the **immune system**), and is aimed essentially at maintaining the genetic integrity of an individual, protecting it from the invasion of substances that can bear the imprint of a foreign genetic code.

The main function of the immune system is *self/non-self discrimination*. This ability to distinguish between self and non-self is necessary to protect the organism from invading pathogens and to eliminate modified or altered cells (e.g. malignant cells). Since pathogens may replicate intracellularly (viruses and some bacteria and parasites) or extracellularly (most bacteria, fungi and parasites), different components of the immune system have evolved to protect against these different types of pathogens. It is important to remember that infection with an organism does not necessarily mean diseases, since the immune system in most cases will be able to eliminate the infection before disease occurs. Disease occurs only when the virulence of the invading organism is great or when immunity is compromised. Although the immune system, for the most part, has beneficial effects, there can be detrimental effects as well. During *inflammation*, which is the response to an invading organism, there may be local discomfort and collateral damage to healthy tissue as a result of the toxic products produced by the immune response.

Host defense mechanisms

The response of the immune system to the introduction of foreign substances is called the **immune response**. **Immunity** is a part of a complex system of defense reactions of the body. These defense reactions can be **innate (non-specific, constitutive)** or **acquired (specific, inducible).**

1. **Constitutive defenses**: Defenses common to all healthy animals. These defenses provide general protection against invasion by normal flora, or colonization, infection, and infectious disease caused by pathogens. The constitutive defenses have also been referred to as *"natural" or "innate" resistance*, since they are inherent to a specific host, but these terms are better reserved for certain types of constitutive defense.

2. **Inducible defenses**: Defense mechanisms that must be induced or turned on by host exposure to a pathogen (as during an infection). Unlike the constitutive defenses, they are not immediately ready to come into play until after the host is appropriately exposed to the parasite. The inducible defenses involve the immune responses to a pathogen causing an infection. The inducible defenses are generally quite specifically directed against an invading pathogen. The constitutive defenses are not so specific, and are directed toward general strategic defense.

Table 17 — Host defenses

Inborn, nonspecific immunity		THIRD LINE OF DEFENCE **Acquired, specific immunity**	
FIRST LINE OF DEFENCE	SECOND LINE OF DEFENCE	**Natural immunity**	**Artificial immunity**
Physical barriers Genetic barriers Chemical barriers	Inflammatory response Interferons Phagocytosis	Active immunity (infection)	Active immunity (vaccination)
		Passive immunity (maternal antibodies)	Passive immunity (immune serum)

Table 18 — Main characteristics of specific (acquired) and non-specific (innate) immunity

Non-specific immunity	**Specific immunity**
Response is **antigen-independent**	Response is **antigen-dependent**
There is **immediate** maximal response	There is a **lag time** between exposure and maximal response
Not antigen-specific	**Antigen-specific**
Exposure results in **no immunologic memory**	Exposure results in **immunologic memory**

NONSPECIFIC IMMUNITY

Constitutive defense

The constitutive defenses of the host can be arranged in the following levels:

SYSTEM LEVEL:
1) Differences in susceptibility to certain pathogen;
2) Anatomical defenses;
3) Microbial antagonism;
4) Inflammation.

CELLULAR LEVEL: Phagocytosis.

HUMORAL LEVEL: Tissue bactericides, including complement.

Differences in susceptibility of animal hosts to microbial pathogens

This type of resistance is also called innate and natural resistance. There are two aspects innate resistance: (1) genetic resistance among all members of a species, called species resistance and (2) individual resistance within the same animal species.

Species resistance: certain animals are naturally resistant or nonsusceptible to certain pathogens. Certain pathogens infect only humans, not lower animals, e.g. syphilis, gonorrhea, measles, poliomyelitis. On the other hand, certain pathogens (e.g. canine distemper virus) do not infect humans. Little information is available to explain these absolute differences in susceptibility to a pathogen but it could be due to: 1) *Absence of specific tissue* or *cellular receptors* for attachment (colonization) by

the pathogen. 2) *Temperature of the host* and ability of pathogen to grow. For example, birds do not normally become infected with mammalian strains of *Mycobacterium tuberculosis* because these strains cannot grow at the high body temperature of birds. 3) *Lack of the exact nutritional requirements* to support the growth of the pathogen. Naturally-requiring purine-dependent strains of *Salmonella typhi* grow only in hosts supplying purines. Mice and rats lack this growth factor and pur-strains are avirulent. By injecting purines into these animals, such that the growth factor requirement for the bacterium is satisfied, the organisms prove virulent. 4) *Lack of a target site* for a microbial toxin.

Individual resistance: there are many reasons why individuals of the same animal species may exhibit greater or lesser susceptibility to the same infective agent.

1. Age*:* Increased susceptibility in the young may, in some instances, is due to hormonal influence. The susceptibility of the vaginal epithelium in prepubertal girls to gonococcal infection is instance of the effect of sex hormones on resistance. Some infections like poliomyelitis and chicken-pox tend to be more severe in adults than in young children, due to hypersensitivity that causes greater tissue damage. Conversely, hepatitis B virus infections in the newborn are usually asymptomatic because clinical disease requires adequate immune response which is lacking at that age. However, the virus multiplies unrestrained and such neonates end up as chronic viral carriers, often developing late hepatic complications. Old persons are highly susceptible to infections due to the waning of their immune responses and other infirmities like enlarged prostate leading to urinary stasis.

2. Sex: usually linked to the presence and/or development of the sex organs. For example, mastitis and infectious diseases leading to abortion will obviously occur only in the female; orchitis would occur only in males.

3. Stress: Stress is a complex of different factors and apparently has a real influence on health. Undue exertion, shock, change in environment, climatic change, nervous or muscular fatigue, etc. are factors known to contribute to increases in susceptibility to infection. The best explanation is that in time of stress the output of cortisone from the adrenal cortex is increased. This suppresses the inflammatory processes of the host and the overall effect may be harmful.

4. Intercurrent disease or trauma: The normal defenses of an animal are impaired by organic diseases such as leukemia, diabetes, AIDS, etc. The high incidence of staphylococcal sepsis in diabetes may be related to the increased level of carbohydrates in tissues. Frequently, inflammatory or immune responses are delayed or suppressed. Influenza may predispose an individual to pneumonia. Smoking tobacco predisposes to infections of the respiratory tract.

5. Therapy against other diseases: Modern therapeutic procedures used in some diseases can render an individual more susceptible to infection. Under these conditions, not only pathogens but organisms of the normal flora and nonpathogens in the host's environment may be able to initiate infection. Examples of therapeutic procedures that reduce the efficiency of the host's defenses are treatment with corticosteroids, cytotoxic drugs, antibiotics, or irradiation. Corticosteroids exert an important influence on the response to infection. They depress the host's resistance by their antiinflammatory and antiphagocytic effects and by the suppression of antibody formation and hypersensitivity. They also have a beneficial effect in that they neutralise the harmful effect of bacterial products such as endotoxins.

Anatomical defenses

Skin: The intact surface of the healthy epidermis seems to be rarely if ever penetrated by bacteria. If the integrity of the epidermis is broken (by the bite of an insect, needle stick, abrasion, cut, etc.) invasive microbes may enter. The normal flora of the skin, which metabolize substances secreted onto the skin, produce end products (e.g. fatty acids) that discourage the colonization of skin by potential pathogens. Perspiration contains lysozyme and other antimicrobial substances (see figure 55).

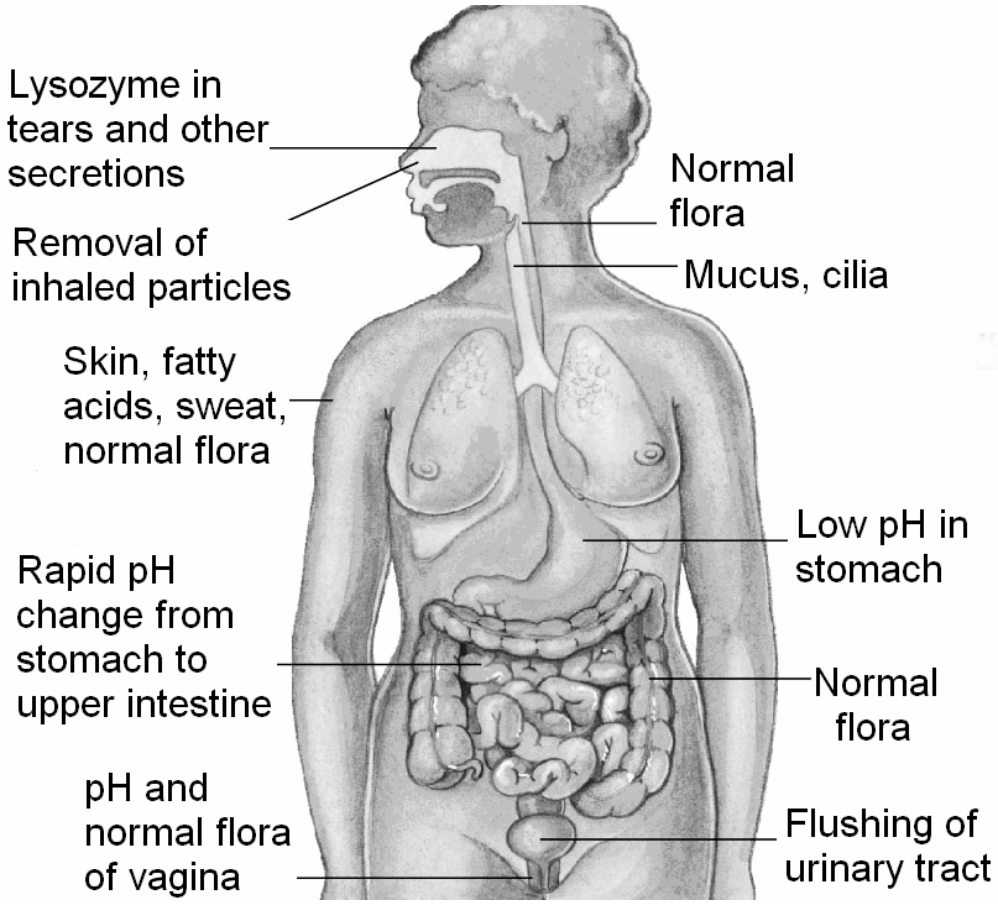


Figure 55 — Anatomical defences

Mucous membranes: Many are heavily colonized with bacteria in whose moist secretions they survive. These normal flora are restricted from entry and usually occupy any attachment sites that might otherwise be used by pathogens. The normal flora established on mucous membranes may antagonize non-indigenous species by other means, as well. Typically, mucus contains a number of types of anti-microbial compounds, including lysozyme and secretory antibodies (IgA).

Respiratory tract: The mucosa of the respiratory tract has several innate mechanisms of defence. The very architecture of the nose prevents entry of microorganisms to a large extent, the inhaled particles being arrested at or near the nasal orifices. Those that pass beyond are held by the mucus lining the epithelium, and are swept back to the pharynx where they tend to be swallowed or coughed out. The cough reflex is an important defence mechanism of the respiratory tract. The cilia on the respiratory epithelial cells propel particles upwards. Na-

sal and respiratory secretions contain mucopolysaccharides capable of combining with influenza and certain other viruses. Particles that manage to reach the pulmonary alveoli are ingested by the phagocytic cells present there.

Mouth, stomach and intestinal tract: Microorganisms entering by the oral route, more than any other, have to compete with the well-adapted normal flora of the mouth and intestine. Most organisms that are swallowed are destroyed by acid and various secretions of the stomach. Alkaline pH of the lower intestine can discourage other organisms. The peristaltic action of the intestine ultimately flushes out organisms which have not succeeded in colonization. Bile salts and lysozyme is present, which kill or inhibit many types of bacteria. **Urogenital Tract:** The flushing mechanisms of sterile urine, and the acidity of urine, maintain the bladder and most of the urethra free of microorganisms. The vaginal epithelium of the female maintains a high population of *Lactobacillus acidophilus* whose acidic end products of metabolism (lactic acid) prevent colonization by most other types of microorganisms including potentially-pathogenic yeast (*Candida albicans*).

Eyes (Conjunctiva): The conjunctiva of the eye is remarkably free of most microorganisms. Blinking mechanically removes microbes, the lavaging action of tears washes the surface of the eye, and lachrymal secretions (tears) contain relatively large amounts of lysozyme.

Inflammation

Of all the defense mechanisms in the animal host, the inflammatory response may be the most important for dealing with microbial infection. Inflammation is necessary for the proper functioning of all the host defenses, including the immune defenses, because it focuses all circulating antimicrobial factors on the site of infection. These include phagocytes, lymphocytes, antibodies, complement and other antimicrobial components of plasma. *Inflammation is a tissue reaction to infection or injury, the characteristic symptoms of which are redness, swelling, heat and pain.* These are sometimes called the **cardinal signs of inflammation**.

Inflammation can be induced by certain **immunological reactions**, **tissue damage**, or the **entry of an injurious agent** (microbial or nonmicrobial). Certain bacterial cells and/or their products (e.g. structural components or toxins) can induce an inflammatory response. Inflammation increases the blood supply and temperature in the inflamed tissues, which favors maximal metabolic activity of the leukocytes, and lowers the pH slightly, which tends to inhibit the multiplication of many microorganisms.

A **rise of temperature** (fever) following infection is a natural defence mechanism and not only helps to accelerate the physiological processes but may, in some cases, actually destroy the infecting pathogens. Therapeutic induction of fever was employed for the destruction of Treponema pallidum in the tissues of syphilitic patients before penicillin became available.

Fever stimulates the production of interferon and aids recovery from viral infections. Infection or injury leads to a sudden increase in plasma concentrations of certain proteins, collectively called acute phase proteins. These include **C reactive protein** (CRP), **mannose binding protein**, **alpha-1-acid glycoprotein** and many others. CRP and some other acute phase proteins activate the alternative pathway of complement. They are believed to enhance host resistance, prevent tissue injury and promote repair of inflammatory lesions.

The overall effect of an inflammatory reaction is to recruit various cells and components to the actual site of microbial invasion. Many of these cells and plasma components have a direct role in defense against the intruding microorganism. These include **neutrophils** (phagocytes which engulf and destroy the microbes); **macrophages** and **lymphocytes** which are the cells necessary to initiate immunological responses against the pathogen; pre-existing **antibodies** which can neutralize microbial pathogens or their toxins; and plasma components such as **lysozyme**, **complement** and **fibrin**, which have a variety of antimicrobial activities.

As a result of infection or tissue damage, complement is activated leading to the release of complement fragments that can activate local mast cells leading to the release of vasoactive chemicals such as *leukotrienes, prostaglandins* and *histamine*. These agents and a range of cytokines are also released by activated tissue macrophages. They have an effect on the local endothelium increasing the expression of a range of adhesion molecules, facilitating the immobilization of leukocytes. Prostaglandins cause blood vessel dilatation and enhance the effects of histamine and bradykinin on vascular permeability. Leukotrienes stimulate the migration of leukocytes into the tissues.

Table 19 — Functions of components in the inflammatory process

Component	Function
Bradykinin, histamine, serotonin, prostaglandins	Inflammatory agents which act on the vascular system to produce increased blood flow and permeability
Fibrin	Coagulates and may localize an invading pathogen
Lysozyme	Causes lysis of bacterial cell walls
Complement	Various activities increase the inflammatory response and lead to increased phagocytosis and complement-mediated lysis of cells
Antibodies	Block colonization by pathogens; neutralize microbial toxins or viruses; opsonize pathogens making them more susceptible to phagocytosis; activate complement
Pyrogens, including endogenous pyrogen (Interleukin 1)	Cause fever acting on the thermo-regulatory control centers in the hypothalamus. (Interleukin-1, which is produced by macrophages, also promotes activation and mitosis of B-cells and T-cells)
Neutrophils	Migrate to focus of infection and ingest and destroy foreign agents by phagocytosis
Macrophages	Engulf and destroy infective agents, process antigenic components and convey them to lymphocytes
Immunocompetent lymphocytes (B-cells and T-cells)	For direct participation in immunological responses (AMI and CMI)

Thus, the different molecules produced during inflammation have different effects but overall are responsible for the induction of pain, fever, vascular permeability and chemotaxis of polymorphonuclear leukocytes (Table). Inflammation is a complex series of biochemical and cellular events leading to the five cardinal signs of heat, pain, swelling, redness and ultimately loss of function. It involves the concerted action of the immune, clotting, fibrinolytic and kinin systems. A range of chemicals such as prostaglandins and leukotrienes are released by cells and affect immune cells and others contributing to a proinflammatory state. Although mast cells and basophils are the principle producers of these inflammatory mediators, other cells such as eosinophils, neutrophils and platelets are also capable of doing so.

Microbial antagonism

This refers to the protection of the surfaces afforded by an intact normal flora in a healthy animal, and it has already been discussed in several contexts. There are three main ways that the normal flora protects the surfaces where they are colonized:

1. **Competition with non-indigenous species** for binding (colonization) sites. The normal flora is highly-adapted to the tissues of their host.

2. **Specific antagonism** against non-indigenous species. Members of the normal flora may produce highly specific proteins called bacteriocins which kill or inhibit other (usually closely-related) species of bacteria.

3. **Nonspecific antagonism** against non-indigenous species. The normal flora produces a variety of metabolites and end products that inhibit other microorganisms.

Phagocytic defenses

Phagocytes are a class of cells which are capable of ingestion (engulfment) and destruction of microorganisms that are responsible for inciting the inflammatory response. First to accumulate around the invaders and initiate the phagocytic process are **neutrophils**. Later, local and blood-borne **macrophages** also migrate to the tissue site and initiate phagocytosis. Neutrophils (also known as polymorphonuclear leucocytes or PMNs) and macrophages are sometimes referred to as professional phagocytes for their roles in this process.

Neutrophils have their origin in multi-potential stem cells in the bone marrow. They differentiate in the marrow and are released in a mature form, containing a full complement of bactericidal agents. They are short-lived cells which constitute 30–70% of the circulating white blood cells (leukocytes). Cytoplasmic granules are formed from the Golgi apparatus. These granules are called **lysosomes** and contain the various bactericidal and digestive enzymes which can destroy bacterial cells after engulfment. The contents of lysosomal granules include lysozyme, cationic proteins, acid hydrolases, proteases, peroxidase and lactoferrin.

Properties of Macrophages (also called **mononuclear phagocytes**) also arise from bone marrow stem cells which give rise to promonocytes which develop into **monocytes** that are released into the blood stream. Monocytes make up 3–7% of the circulating white blood cells. The monocyte is actively phagocytic and bactericidal. Within 2 days or so, the blood stream monocytes emigrate into the tissues where they settle down, enlarge and become fixed macrophages, which also have phagocytic potential. Macrophages are more active in phagocytosis than monocytes and develop many more granules containing hydrolytic enzymes. New macrophages can develop by cell division under inflammatory stimuli, but most macrophages are matured blood monocytes.

The total pool of macrophages is referred to as the **system of mononuclear phagocytes**. The system is scattered throughout connective tissue, basement membranes of small blood vessels, liver sinusoids, the spleen, lung, bone marrow and lymph nodes. Monocytes from the blood migrate into virtually every organ in the body where they mature into fixed macrophages. In the lymph nodes, they function as scavengers to remove foreign material from the circulation.

The **phagocytic process** involves the following sequence of events:

1. Delivery of phagocytic cells, monocytes or neutrophils, to the site of microbial infection involves two processes: **Diapedisis**: the migration of cells across vascular walls which is initiated by the mediators of inflammation (histamine, prostaglandins,

etc.) **Chemotaxis.** Phagocytes are motile by ameboid action. Chemotaxis is movement of the cells in response to a chemical stimulus. A number of chemotactic factors (**chemoattractants**) have been identified, both for neutrophils and monocytes. These include bacterial products, cell and tissue debris, and components of the inflammatory exudate derived from complement (**C3a and C5a**). These chemoattractants attract phagocytes to microbes to be injected (see figure 56).

2. Phagocytic adherence this step usually involves several types of surface receptors on the phagocyte membrane. The major receptors on phagocytes recognize the Fc-portion of IgG. Another receptor binds a complement factor C3b. Under certain circumstances of infection, bacteria or viruses may become coated or otherwise display on their surfaces one or another of these substances (i.e., IgG, C3b, fibronectin or mannose). Such microbes are said to be **opsonized** and such substances as **IgG** or **complement C3b** bound to the surface of microbes are called **opsonins**. Opsonins provide extrinsic ligands for specific receptors on the phagocyte membrane, which dramatically increases the rate of adherence and ingestion of the pathogen.

3. Ingestion After attachment of the phagocyte to its target, some sort of signal generation, which is poorly understood, results in physical or chemical changes in the cell that triggers ingestion. Ingestion is an engulfment process that involves invagination of the cell membrane enclosing the particle and ultimately releasing it into the cytoplasm of the cell within a membrane vesicle. The end result of ingestion is entry of the particle enclosed in a vesicle derived from the plasma membrane of the cell. This structure is called the **phagosome**.

4. Formation of the phagolysosome the phagosome migrates into the cytoplasm and collides with lysosomal granules which explosively discharge their contents into the membrane-enclosed vesicle (phagosome). Membranes of the phagosome and lysosome actually fuse resulting in a digestive vacuole called the **phagolysosome.** Other lysosomes will fuse with the phagolysosome. It is within the phagolysosome that killing and digestion of the engulfed microbe takes place. Some of the microbicidal constituents of the lysosomes of neutrophils and macrophages include lysozyme, cationic proteins, various proteases and hydrolyases and peroxidases.

5. Intracellular killing of organisms after phagolysosome formation the first detectable effect on bacterial physiology, occurring within a few minutes after engulfment, is loss of viability (ability to reproduce). The exact mechanism is unknown. Inhibition of macromolecular synthesis occurs later. By 10 to 30 minutes after ingestion many pathogenic and nonpathogenic bacteria are killed followed by lysis and digestion of the bacteria by lysosomal enzymes.

6. Intracellular digestion Dead microbes are rapidly degraded in phagolysosomes to low molecular-weight components. Various hydrolytic enzymes are involved including lysozyme, proteases, lipases, nucleases, and glycosylases. Neutrophils die and lyse after extended phagocytosis, killing, and digestion of bacterial cells. This makes up the characteristic properties of pus.

7. Contents of phagolysosome eliminated by exocytosis.

The microbicidal activities of phagocytes are usually divided into **oxygen-dependent** and **oxygen-independent** events.

Oxygen-independent activity: Lysosomal granules contain a variety of extremely basic proteins that strongly inhibit bacteria, yeasts and even some viruses. A few molecules of any one of these cationic proteins appear able to inactivate a bacte-

rial cell by damage to their permeability barriers, but their exact modes of action are not known. The lysosomal granules of neutrophils contain **lactoferrin**, an extremely powerful iron-chelating agent, which binds potential iron needed for bacterial growth. The pH of the phagolysosome may be as low as 4 due to accumulation of lactic acid, which is sufficiently acidic to prevent the growth of most pathogens.

Oxygen-dependent activity: Liganding of Fc receptors (on neutrophils, monocytes or macrophages) and mannose receptors (on macrophages) increases their O_2 uptake, called the **respiratory burst**. These receptors activate a membrane-bound **NADPH oxidase** that reduces O_2 to O_2^- (superoxide). Superoxide can be reduced to $OH^\cdot$ (hydroxyl radical) or dismutated to H_2O_2 (hydrogen peroxide) by superoxide dismutase. O_2^-, $OH^\cdot$, and H_2O_2 are activated oxygen species that are potent oxidizing agents in biological systems which adversely affect a number of cellular structures including membranes and nucleic acids.

Macrophages egest digested debris and allow insertion of microbial antigenic components into the plasma membrane for **presentation** to lymphocytes in the immunological response.

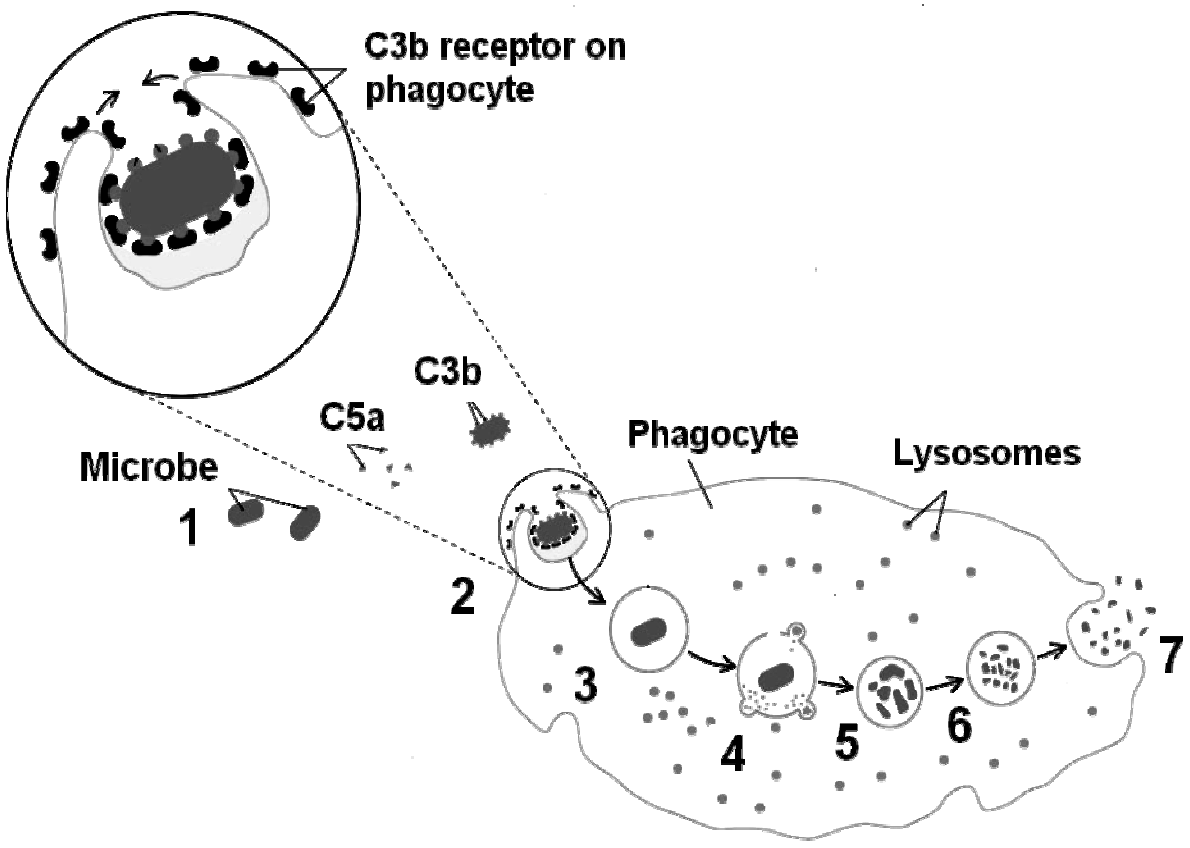


Figure 56 — Phagocytic process

Bacteria can avoid the attention of phagocytes in a number of ways

1. Pathogens may invade or remain confined in **regions inaccessible to phagocytes** (e.g. the lumens of glands, the urinary bladder are not patrolled by phagocytes).

2. Some pathogens are able to avoid provoking an **overwhelming inflammatory response**. Without inflammation the host is unable to focus the phagocytic defenses.

3. Some bacteria or their products **inhibit phagocyte chemotaxis**. Fractions of Mycobacterium tuberculosis are known to inhibit leukocyte migration.

4. Some pathogens can cover the surface of the bacterial cell with a component which is seen as "self" by the host phagocytes and immune system. Such a strategy **hides the antigenic surface** of the bacterial cell. For example, pathogenic Staphylococcus aureus produces cell-bound coagulase which clots fibrin on the bacterial surface.

5. Classical examples of antiphagocytic substances on the bacterial surface include: **polysaccharide capsules** of *S. pneumoniae*, *Treponema pallidum* and *Klebsiella pneumoniae,* **fimbriae** of Group A streptococci, **O-polysaccharide** associated with LPS of *E. coli*, **K-antigen** (acidic polysaccharides) of *E. coli* or the analogous **Vi-antigen** of *Salmonella Typhi*.

Some bacteria survive inside of phagocytic cells, in either neutrophils or macrophages. Bacteria that can resist killing and survive or multiply inside of phagocytes are considered **intracellular parasites**. In this case, the environment of the phagocyte may be a protective one, protecting the bacteria during the early stages of infection or until they develop a full complement of virulence factors. The intracellular environment protects the bacteria against the activities of extracellular bactericides, antibodies, drugs, etc. Some bacteria, such as brucella and lepra bacilli, resist intracellular digestion and may actively multiply inside the phagocytic cells. Phagocytosis in such instances may actually help to disseminate infection to different parts of the body.

Antimicrobial substances in host tissues

The body fluids and organized tissues of animals naturally contain a variety of antimicrobial agent that kill or inhibit the growth of microbes.

Table 20 — Antimicrobial substances of the host origin present in body fluid and organized tissues

Substance	Common source	Activity
Complement	Serum	Cell death or lysis of bacteria; participates in inflammation; phagocytosis of bacteria
Lysozyme	Serum, saliva	Bacterial cell lysis
Peroxidase	Saliva, tissues	Act with peroxide to cause lethal oxidations of cells
Interferons (α, β)	Virus-infected cells, T- lymphocytes	Resistance to virus infections
Basic proteins and polypeptides (histones, ß-lysins and tissue polypeptides)	Serum or organized tissues	Disruption of bacterial plasma membrane
Interleukins	Macrophages, lymphocytes	Cause fever; promote activation of immune system

Complement

Complement can be considered as part of the constitutive host defense mechanisms (it is present at constitutive levels) because of its role in inflammation and phagocytosis. However, the antimicrobial activities of complement can be activated completely by reactions between antigens and antibodies and, therefore, it may play a role in the inducible (immune) defenses, as well. Complement is an enzymatic system of serum proteins that are sequentially activated in many Ag–Ab reactions resulting in disruption of membranes. Therefore, complement (C') may be involved in the lysis of certain bacteria,

some viruses, and other microorganisms. In addition, some C' components play a part in phagocytic chemotaxis, opsonization and the inflammatory response.

The complement system consists of at least 20 chemically and immunologically distinct serum proteins comprising the **complement components**, the **properdin system** and the **control proteins**.

Complement is normally present in the body in an inactive form but when its activity is induced **by antigen-antibody combination** or other stimuli, C components react in a specific sequence as a **cascade**. Basically, the *C cascade is a series of reactions in which the preceding components act as enzymes on the succeeding components, cleaving them into dissimilar fragments*. The larger fragments usually join the cascade. The smaller fragments which are released often possess biological effects which contribute to defence mechanisms by amplifying the inflammatory process, increasing vascular permeability, inducing smooth muscle contraction, causing chemotaxis of leucocytes, promoting virus neutralization, detoxifying endotoxins and effecting the release of histamine from mast cells.

The C cascade can be triggered off by two parallel but independent mechanisms or pathways which differ only in the initial steps. Once C3 activation occurs, the subsequent steps are common in both pathways, which have been called the **classical C pathway** and **the alternative or properdin pathway**.

The classical pathway is so called because it was the first one identified. But actually it is a more recently evolved mechanism of specific active immunity, while the alternative pathway represents a more primitive system of nonspecific innate immunity.

Complement is activated in the **classical pathway** by reactions between antibodies and antigens on the surface of a microbe. Some Immunoglobulins (i.e., IgG and IgM) can "fix complement" because they have a complement binding site on the Fc-portion of the molecule.

The classical pathway consists of the following steps:

1. The **first step** is the binding of C1-unit to the antigen-antibody complex (traditionally represented as EA). The recognition unit of C1 is subunit C1q, which reacts with the Fc-piece of bound molecule of IgM or two molecules of IgG can therefore initiate the process. C1q binding in the presence of calcium ions leads to sequential activation of C1r and C1s (see figure 57).

2. Activated C1s-subunit is an **esterase**, one molecule of which can cleave several molecules of C4. C4 is split into **C4a**, which is an **anaphylatoxin**, and C4b which binds to cell membranes along with C1.

3. C14b in the presence of magnesium ions cleaves C2 into C2a, which remains linked to cell-bound C4b, and C2b which is released into fluid phase. C4b2a has enzymatic activity and is referred to as the classical pathway **C3-convertase**.

4. C3-convertase splits C3 into two fragments — C3a which is an anaphylatoxin, and C3b which remains cell-bound along with **C4b2a** to form a complex **C4b2a3b** which has enzymatic activity and is called **C5-convertase**.

C5-convertase cleaves C5 into **C5a**, an **anaphylatoxin** which is released into the medium, and C5b which continues with the cascade. C6 and C7 then join together. A heat stable **complex C567** is formed, part of which binds to the cell membrane and prepares it for lysis by C8 and C9 which join the reaction subsequently. Most of C567 escape and serve to amplify the reaction by adsorbing on the bacterial cells and rendering them susceptible to lysis by C8 and C9.

The mechanism of **complement mediated cytolysis** is the production of “holes”, approximately 100 A in diameter on the cell membrane. This disrupts the osmotic integrity of the membrane, leading to the release of the cell contents (figure 58).

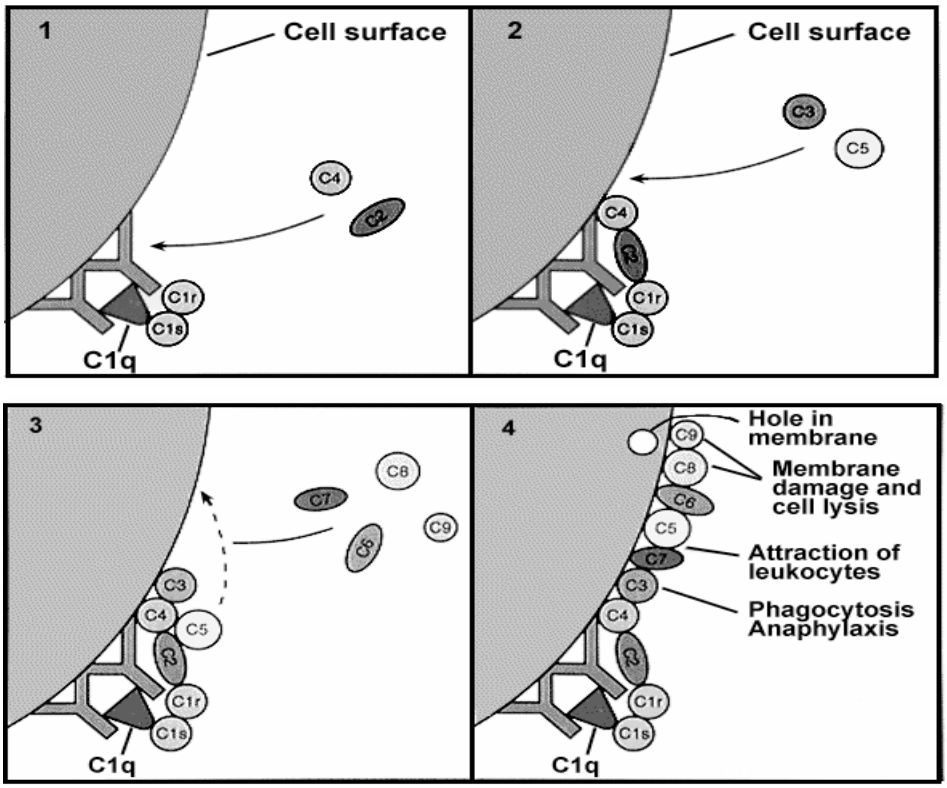


Figure 57 — Scheme of complement activation

In addition to the classical pathway» of complement activation an **alternative pathway** (sometimes called the "properdin pathway") of complement activation exists which is independent of immunoglobulins. Insoluble polysaccharides (including bacterial LPS, peptidoglycan and teichoic acids) can activate complement. This allows antibody-independent activation of the complement cascade that may be important in initial (pre-antibody) defense against various types of infections caused by bacteria.

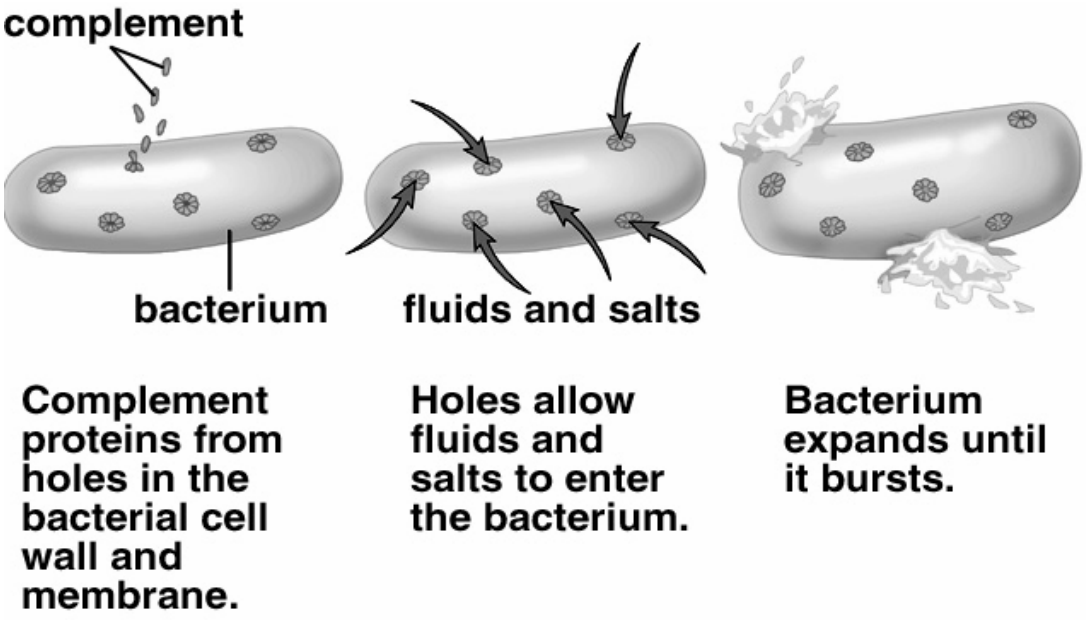


Figure 58 — Mechanism of complement mediated cytolysis

SPECIFIC IMMUNITY

Inducible defenses (acquired immunity)

The **inducible defenses** are so-called because they are induced upon primary exposure to a pathogen or one of its products. The inducible defenses are a function of the **immunological system** and the **immune responses**. They must be triggered in a host and initially take time to develop. The type of resistance thus developed in the host is called **acquired immunity** (*which occurs after exposure to an antigen is specific and is mediated by either antibody or lymphoid cells*).

Acquired (adaptive) immunity may be divided into two types based on how it is acquired by the host:

1. **Active immunity** is induced after contact with foreign antigens (eg, microbes or their products). This contact may consist: a) clinical infection; b) immunization with live or killed infectious agents or their antigens; c) exposure to microbial products (toxins, toxoids); d) transplantation of foreign cells. In all these instances the host actively produces antibodies and lymphoid cells acquire the ability to respond to the antigens**. Thus,** antibodies are produced by the host in response to the infectious agent itself (e.g. recovery from the disease), or in response to vaccination with some product derived from the infectious agent. **Advantages** of active immunity include *long-term resistance* and the capacity to respond faster and to a greater extent on subsequent contact with the same antigen. **Disadvantages** include the slow onset of resistance and the need for prolonged or repeated contact with antigen.

2. **Passive immunity** is transmitted by antibodies or lymphocytes preformed in another host. The passive administration of antibody against certain viruses can be useful during incubatory period to limit viral multiplication. Also examples of passive immunity are placental transfer of antibodies from mother to fetus and transfer of antibodies from mother to infant in milk by nursing. The main **advantage** of passive immunization with preformed antibodies is the prompt availability of large amount of antibodies. **Disadvantages** are the shot life span of these antibodies and possible hypersensitivity reactions if antibodies from another species administered.

In either case of active or passive immunity, resistance may be acquired by **natural** means or by **artificial** means. *Artificial active immunity* is the resistance induced by vaccines. **Vaccines** are preparations of live or killed microorganisms or their products used for immunization.

Vaccines

Effective vaccines *are:* 1. Safe; 2. Protective for sustained period; 3. Induce neutralising antibody. ***In addition they should be:*** 1. Biologically stable; 2. Cheap to produce; 3. Easy to administer

Vaccination is intended to provide long-term protection after its administration. Effector T- and B-cells last only a few days, so the prime requisite of any vaccine is to generate immunological memory. Successful vaccines: activate antigen-presenting cells to initiate antigen processing and produce cytokines activate both T and B-cells to give a high yield of memory cells generate T helper and T cytotoxic cells to several epitopes, to overcome the variation in the immune response in the population due to MHC polymorphism enable the persistence of antigen, probably on follicular dendritic cells in lymphoid tissue, to elicit continued production of antibody from B-cells.

Examples of vaccines are as follows:

1. **Bacterial vaccines:**
   a. Live (BCG vaccine for tuberculosis)
   b. Killed (Cholera vaccine)
   c. Subunit (Typhoid Vi-antigen)
   d. Bacterial products (Tetanus toxoid)
2. **Viral vaccines:**
   a. Live (Oral polio vaccine-Sabin)
   b. Killed (Injectable polio vaccine-Salk)
   c. Subunit (Hepatitis B vaccine).

Live vaccines initiate an infection without causing any injury or disease. The immunity lasts for several years but booster doses may be necessary. Live vaccines may be administered orally or parenterally. Killed vaccines are generally less immunogenic than live vaccines, and protection lasts only for a short period. They have, therefore, to be administered repeatedly. Killed vaccines may be given orally but this route is generally not effective. Parenteral administration provides humoral antibody response, which may be improved by the addition of "adjuvants" (for example, aluminium phosphate).

Artificial passive immunity is the resistance passively transferred to a recipient by the administration of antibodies. The agents used for this purpose are hyperimmune sera of animal or human origin and pooled human gamma globulin. These are used for prophylaxis and therapy. Equine hyperimmune sera such as *antitetanus serum* prepared from hyperimmunised horses used to be extensively employed. They gave temporary protection but carried the disadvantages of hypersensitivity and immune elimination. Human hyperimmune globulin (for example, tetanus immune globulin, TIG) is free from those complications and also gives more lasting protection.

A special type of immunization is the injection of immunologically competent lymphocytes. This is known as *adoptive immunity* and does not have general application. Instead of whole lymphocytes, an extract of immunologically competent lymphocytes, known as the "transfer factor", can be used. This has been attempted in the treatment of certain types of diseases (for example, leprosy).

Measurement of immunity

The truly valid measurement of immunity is to *test the* ***resistance of an individual to a challenge by the pathogen***. This is, however, not applicable since the challenge itself alters the state of immunity. It is, therefore, not possible to measure accurately the level of immunity in an individual. Estimates of immunity are generally made by statistical methods using large numbers of individuals.

A simple method of testing immunity is to relate its level to some convenient indicator, such as ***demonstration of the specific antibody***. This is not always reliable as the immune response to a pathogen consists of the formation of antibodies to several antigens present in it, as also to the production of cellular immunity. The antibodies may be demonstrated by a variety of techniques such as agglutination, precipitation, complement fixation, hemagglutination, neutralization, ELISA and others. In some instances, as in diphtheria where pathogenesis is due to a well defined antigen (the toxin), the level of immunity can be assayed by in vitro or in vivo (**Schick test**) methods. Where protection is associated with cell mediated immunity, **skin tests** for delayed hypersensitivity and in vitro tests for CMI afford an indication of immunity.

Antigens

An **antigen** has been defined as *any substance which, when introduced parenterally into the body, stimulates the production of an antibody with which it reacts specifically*. Some antigens may not induce antibodies but may sensitize specific lymphocytes leading to cell-mediated immunity or may cause immunological tolerance.

An **immunogen** is any molecule (or group of molecules) that can induce an immune response, while an **antigen** is any substance that can react with antigenspecific receptors found on the surface of certain white blood cells. Thus, an antigen differs from an immunogen in that although an antigen can interact in a specific way with the immune system, it cannot by itself stimulate an immune response; other stimuli are required. Thus, all immunogens are antigens but not all antigens are immunogens. The word “parenteral” (meaning, outside the itestinal tract) is used in the definition because orally administered antigens are usually hydrolyzed by digestive enzymes and their antigenicity destroyed, so that no antibody formation takes place. When given parenterally, antigens do not undergo any such inactivation and can induce antibody production. However, there are exceptions and some antigens can be immunogenic when given orally, such as *oral vaccines*.

The word “**specifically**”in the definition is important as specificity is the hallmark of all immunological reactions. An *antigen introduced into the body reacts only with those particular B- or T- lymphocytes which carry the specific marker for that antigen* and which produce an antibody or cells complementary to that antigen only. The *antibody so produced will react only with that particular antigen and with no other* — though immunological cross reaction may occur between closely related antigens.

The two attributes of antigenicity are 1) induction of an immune response (**immunogenicity**), and 2) specific reaction with antibodies or sensitized cells (**immunological reactivity**).

Based on the ability of antigens to carry out these two functions, they may be classified into different types. A **complete antigen** is able to induce antibody formation and produce a specific reaction with the antibody so produced. **Haptens** are substances which are incapable of inducing antibody formation by themselves but can react specifically with antibodies. Haptens become immunogenic (capable of inducing antibodies) on combining with a larger molecule *carrier.*

The smallest unit of antigenicity is known as the *antigenic determinant* or **epitope** (see figure 59). The epitope is that small area on the antigen, usually consisting of five aminoacid or sugars in size and that is capable of binding to an antibody. An antigen can have one or more determinants. The combining area on the antibody molecule, corresponding to the epitope, is called the **paratope.**

Superantigens

1. Name given to proteins produced by many pathogens, including bacteria, mycoplasma and viruses that are capable of stimulating large numbers of T-cells.

2. Most important are the bacterial toxins, especially staphylococcal enterotoxins, each of which can bind to the variable region of the beta chain of the T-cell receptor.

3. Activation of T-cells also requires that the superantigen bind to class II MHC molecules on the surface of antigen presenting cells; however they are not processed and presented by APC. Rather superantigens function as intact molecules.

4. Each superantigen can bind one or a few of the different Vbeta regions, of which there are 20–50 in human, so a superantigen can activate large numbers of T cells.

5. Pathology due to large amounts of cytokines: produced by such a high frequency of activated T-cells causing effects such as "septic shock" or "endotoxin shock".

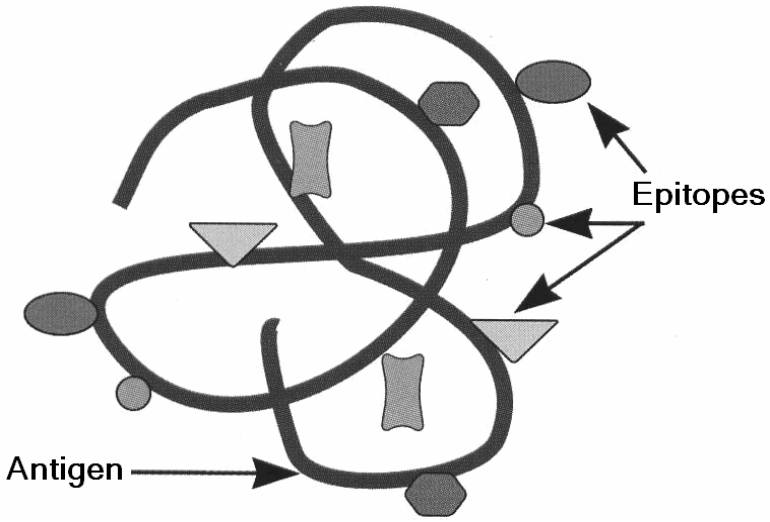


Figure 59 — Epitopes of antigen

Epitopes and paratopes determine the specificity of immunological reactions! Antigens such as bacteria or viruses carry many different types of epitopes, presenting an **antigenic mosaic**. The presence of the same or similar epitopes on different antigens accounts for one type of **antigenic cross reaction**.

Determinants of antigenicity

1. Size: The most potent antigens are usually large proteins. Generally, molecules with a molecular weight less than 5 000 are weakly immunogenic, and very small ones (aminoacids) are nonimmunogenic. Certain small molecules (Haptens) become immunogenic only when linked to a carrier protein.

2. Chemical nature: Most naturally occurring antigens are proteins and polysaccharides. Lipids and nucleic acids are less antigenic. Their antigenicity is enhanced by combination with proteins. A certain degree of structural diversity is required for antigenicity. However, not all proteins are antigenic. A well known exception is gelatin, which is nonimmunogenic because of its structural unstability.

3. Susceptibility to tissue enzymes*:* Only substances which are metabolized and are susceptible to the action of tissue enzymes behave as antigens. Phagocytosis and intracellular enzymes appear to play an essential role in breaking down antigens into immunogenic fragments. Substances unsusceptible to tissue enzymes are not antigenic. Substances very rapidly broken down by tissue enzymes are also not antigenic.

4. Foreignness*:* Only antigens which are 'foreign' to the individual (nonself) induce an immune response. The animal body contains numerous antigens which induce an immune response when introduced into another individual or species. Molecules recognized as “self” are not immunogenic. Tolerance of self antigens is conditioned by contact with them during the development of the immune apparatus. Breakdown of this homeostatic mechanism results in autoimmunisation and autoimmune disease.

5. Antigenic specificity: The specificity of natural tissue antigens of animals may be considered under different categories as species, iso-, auto- and organ specificities.

a) *Species specificity:* Tissues of all individuals in a species contain ***species specific antigens***. There exists some degree of cross reaction between antigens from related species. Phylogenetic relationships are reflected in the extent of cross reaction between antigens from different species that cause hypersensitivity.

b) Isospecificity: ***Isoantigens*** are antigens found in some but not all members of a species. A species may be grouped depending on the presence of different isoantigens in its members. The best examples of isoantigens are the human erythrocyte antigens based on which individuals can be classified into different blood groups. These are genetically determined. They are of clinical importance in blood transfusion and in isoimmunisation during pregnancy. ***Histocompatibility antigens*** are those cellular determinants specific to each individual of a species. They are recognised by genetically different individuals of the same species when attempts are made to transfer or transplant cellular material from one individual to another.

c) *Autospecificity:* ***Self antigens*** are ordinarily nonantigenic but there are exceptions.

d) *Organ specificity:* Some organs, such as the brain, kidney and lens protein of different species, share the same antigen. Such antigens, characteristic of an organ or tissue and found in different species, are called ***organ specific antigens***.

e) *Heterogenetic specificity:* The same or closely related antigens may sometimes occur in different biological species, classes and kingdoms. These are known as ***heterogenetic antigens***, best exemplified by the Forssman antigen which is a lipid carbohydrate complex widely distributed in many animals, birds, plants and bacteria. It is absent in rabbits, so ***anti-Forssman antibody*** can be prepared in these animals.

Biological classes of antigens

Depending on their ability to induce antibody formation, antigens are classified as T-cell dependent and T-cell independent antigens. Antibody production is the property of B-lymphocytes. For the full expression of this function the *cooperation of T lymphocytes is necessary*. Some antigens can directly stimulate antibody production by B-cells, without the apparent participation of T-cells. Such antigens are called **T-cell independent antigens**. Others that require T-cell participation to generate an immune response are called **T-cell dependent antigens**.

T-cell independent antigens are structurally simple, as in the case of the *pneumococcal capsular polysaccharide, bacterial lipopolysaccharides and the flagellar protein flagellin*. Their immune response is critically dose dependent. Too little is nonimmunogenic, while too much results in immunological tolerance rather than immunity. Their antibody response is usually limited to IgM and IgG3. They do not produce immunological memory. T-cell dependent antigens, on the other hand, are structurally more complex, such as *erythrocytes, serum proteins* and a variety of *protein-hapten complexes*. They are immunogenic over a wide dose range and do not cause tolerance readily. They induce the full set of immunoglobulin — IgM, IgG, IgA and IgE. They produce immunological memory.

Major histocompatibility complex

The membrane of a cell is composed of a lipid bilayer, which has proteins, glycoproteins and other compound molecules inserted in it. Such molecules may act as antigens when introduced to a foreign host (cell surface antigens). Probably the best example of this is the blood group antigens A, B and O. An individual of blood group A will have erythrocytes which express this antigen (A+). If this individual is given group B blood (which expresses the B antigen B+), their immune system will recognise the B antigen as foreign and will destroy the transfused blood. This is why it is vital to cross-match blood.

All other cells of the body have a variety of surface antigens, the nature of which are determined by the *genetic make-up of the host*. These tissue antigens are quite

distinct in each individual and when an organ is transplanted, the donor must be matched to the recipient. If the tissues are not matched, the recipient's immune system will recognise the donor tissue antigens as foreign and will destroy the transplant.

These **human leukocyte antigens** (HLA) are the *molecules that are identified when an individual is 'tissue-typed*'. They are the products of a group of genes known as the **major histocompatibility complex** (MHC).

Antigens coded for by MHC Class I genes are found on the surface of all *nucleated cells* and *platelets*. The antigenically distinct molecules are coded for by different regions within the Class I genome (on chromosome 6).

The MHC Class II molecules HLA-DR, -DP and -DQ are expressed on **antigen presenting cells** (e.g. dendritic cells, macrophages and B-cells), which stimulate an immune response. However, under appropriate conditions, cells that do not normally express them can be induced to do so (e.g. activated T-cells). The level of expression of these molecules may be regulated by cytokines and infectious agents such as some viruses.

Antigen recognition molecules

In order for the immune system to respond to nonself (foreign antigen) a recognition system capable of distinguishing self from nonself had to evolve.

The humoral basis of immunity was established by the demonstration that following the introduction of an antigen into an animal, certain substances called **antibodies** appeared in the serum and tissue fluids, and reacted with the antigen specifically and in some observable manner. Sera having high antibody levels following infection or immunisation were called "immune sera".

The term gammaglobulin thereafter became synonymous with "antibody". Later, many antibodies, such as equine antitoxins, were found to migrate as beta or even alpha globulins. Immunoglobulins provide a structural and chemical concept, while the term "antibody" is a biological and functional concept. *All antibodies are immunoglobulins, but all immunoglobulins may not be antibodies*.

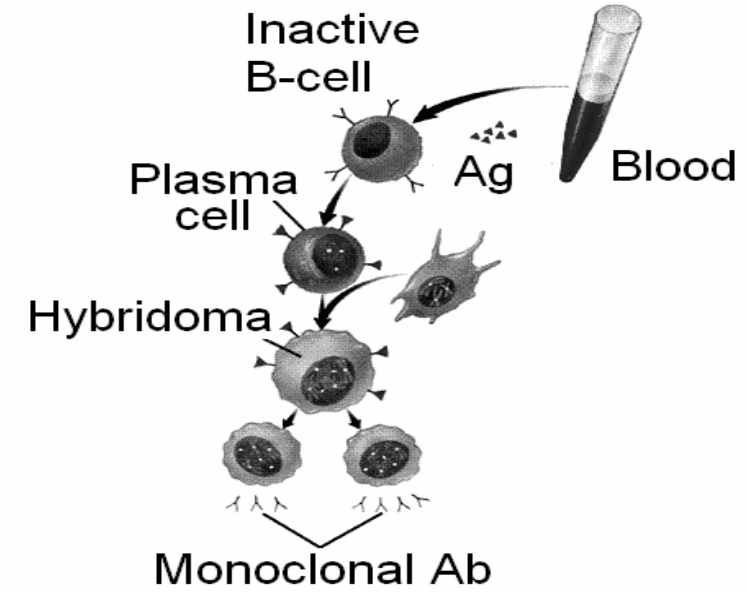
The term "immunoglobulin" is often used interchangeably with "antibody". We will use the term "immunoglobulin" to describe *any* antibody, regardless of specificity, and the term "antibody" to describe an *antigen-specific* "immunoglobulin". Immunoglobulins (Ig) come in different forms (IgA, IgD, IgE, IgG, and IgM) that reflect their structure.

Structure of antibody

Antibodies **(immunoglobulins)** are formed by B-lymphocytes. Each individual has a large pool of different B-lymphocytes (about 10^{11}) that have a life span of days or weeks and are found in the bone marrow, lymph nodes, and gut-associated lymphoid tissues (eg, tonsils or appendix).

B-cells display immunoglobulin molecules (10^5 cell) on their surface. These immunoglobulins serve as receptors for a specific antigen, so that each B-cell can respond to only one antigen or a closely related group of antigens. All immature B-cells carry IgM immunoglobulins on their surface, and most also carry IgD. B cells also have surface receptors for the Fc-portion of immunoglobulins and for several complement components.

An antigen interacts with the B-lymphocyte that shows the best "fit" by virtue of its immunoglobulin surface receptor. The antigen binds to this receptor, and the B-cell is stimulated to divide and form a clone **(clonal selection).** Such selected B-cells soon become **plasma cells** and secrete antibody (see figure 60).



Antibodies are immunoglobulins which react specifically with the antigen that stimulated their production. They make up about 20% of plasma proteins. Antibodies that arise in an animal in response to a single complex antigen are heterogeneous because they are formed by several different clones of cells, each expressing an antibody capable of reacting with a different antigenic determinant on the complex antigen. These antibodies are said to be **polyclonal.**

Figure 60 — Scheme of production of human antibodies

Antibodies that arise from a single clone of cells, eg, in a plasma cell tumor (myeloma), are homogeneous and are referred to as **monoclonal.** Monoclonal antibodies can be produced by fusing a *myeloma cell* with an antibody-producing lymphocyte. Such **hybridomas** produce virtually unlimited quantities of monoclonal antibodies in vitro. Important information about the structure and function of antibodies has been derived from the study of monoclonal antibodies.

All immunoglobulin molecules are made up of light and heavy polypeptide chains. The terms light and heavy refer to molecular weight. Light chains have a molecular weight of approximately 25,000, whereas heavy chains have a molecular weight of approximately 50,000 (see figure 61). **Light (L) chains** are of one of two types, **κ** (kappa) or **λ** (lambda); classification is made based on amino acid differences in their constant regions. Both types occur in all classes of immunoglobulins (IgG, IgM, IgA, IgE, and IgD), but any one immunoglobulin molecule contains only one type of L chain. The amino terminal portion of each L chain contains part of the **antigen-binding site**. **Heavy (H) chains** are distinct for each of the five immunoglobulin classes and are designated **y** (gamma), **μ** (mu), **α** (alpha), **δ** (delta), and **ε** (epsilon). The amino terminal portion of each H chain participates in the antigen-binding site; the other (carboxyl) terminal forms the Fc fragment, which has various biologic activities (eg, complement activation and binding to cell surface receptors).

An individual antibody molecule always consists of identical H chains and identical L chains. The simplest antibody molecule has a Y-shape (Figure 68) and consists of four polypeptide chains: two H chains and two L chains. The four chains are covalently linked by **disulfide bonds**. If such an antibody molecule is treated with a proteolytic enzyme (eg, **papain**), peptide bonds in the **hinge region** are broken. This breakage produces two identical **Fab-fragments**, which carry the antigen-binding sites, and one **Fc-fragment**, which is involved in placental transfer, complement fixation, attachment for various cells, and other biologic activities. L and H-chains are subdivided into **variable regions** and **constant regions.** The regions are composed of repeating segments called **domains**. The structure of these domains has been de-

termined at high resolution by x-ray crystallography. An L-chain consists of one *variable domain* (V_L) and one *constant domain* (C_L). Most H-chains consist of one variable domain (V_H) and three constant domains (C_H). Each domain is approximately 110 amino acids long. Variable regions are responsible for antigen binding; constant regions are responsible for biologic functions.

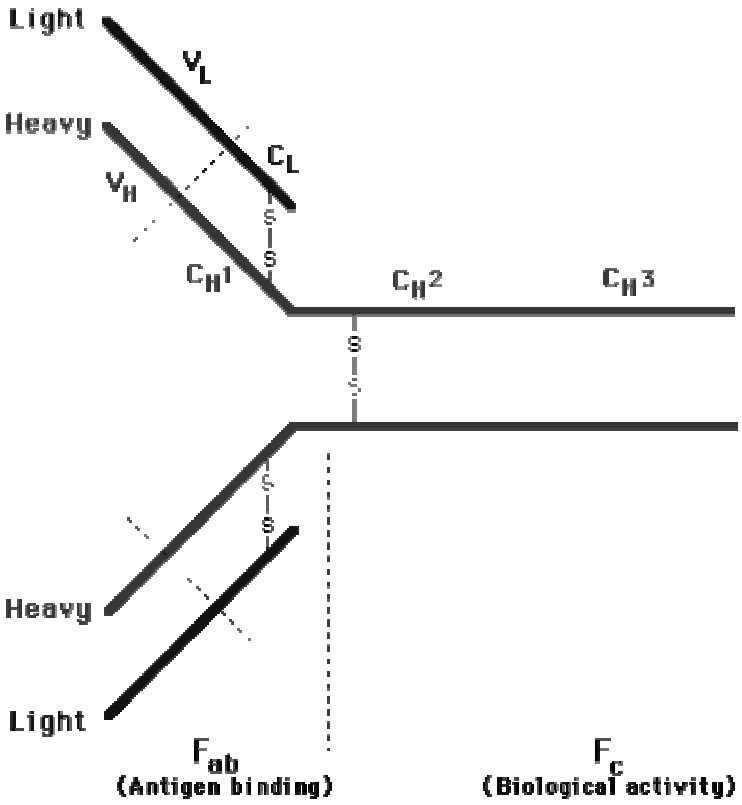


Figure 61 — Structure of antibody (IgG)

Immunoglobulin classes

Human sera contain IgG, IgA, IgM, IgD and IgE in the descending order of concentration. Table 17 shows their characteristics.

IgG: This is the major serum immunoglobulin, constituting about 80 per cent of the total. It has a half life of approximately 23 days. The catabolism of IgG is unique in that it varies with its serum concentration. The normal serum concentration of IgG is about 8–16 mg per ml.

IgG is the only maternal immunoglobulin that is *normally transported across the placenta* and provides *natural passive immunity* in the newborn. It is not synthesised by the fetus in any significant amount. IgG binds to microorganisms and enhances their phagocytosis (opsonisation). Extracellular killing of target cells coated with IgG antibody is mediated through recognition of the surface Fc-fragment by K-cells bearing the appropriate receptors. IgG participates in most immunological reactions such as complement fixation, precipitation, and neutralization of toxins and viruses. It may be considered a general purpose antibody, protective against those infectious agents which are active in the blood and tissues. With most antigens, IgG is a late antibody and makes its appearance after the initial immune response which is IgM in nature.

Four subclasses of IgG have been recognised (IgG 1, IgG2, IgG3, IgG4), each possessing a distinct type of gamma-chain, identifiable with specific antis-era.

IgA: IgA is the second most abundant class, constituting about 10–13 per cent of serum immunoglobulins. The normal serum level is 0.6–4.2 mg per ml. It has a half life of 6–8 days. It is the major immunoglobulin in the milk, saliva and tears.

IgA occurs in two forms. ***Serum* IgA** is principally amonomeric. IgA found on mucosal surfaces and in secretions is a dimer formed by two monomer units joined together by a glycopeptide termed the *J-chain* (J for joining). This is called the ***secretory IgA*** (SIgA).

SIgA is not produced by lymphoid cells but by mucosal or glandular epithelial cells. The secretory piece is believed to protect IgA from denaturation by bacterial proteases in sites such as the intestinal mucosa which have a rich and varied bacterial flora.

SIgA is selectively concentrated in secretions and on mucus surfaces and is believed to play an important role in *local immunity against respiratory and intestinal pathogens.* Secretory IgA is relatively resistant to the digestive enzymes and reducing agents. IgA antibodies may function by inhibiting the adherence of microorganisms to the surface of mucosal cells by covering the organisms and thereby preventing their entry into body tissues. IgA does not fix complement but can activate the alternative complement pathway. It promotes phagocytosis and intracellular killing of microorganisms (see figure 62).

IgM: IgM constitutes 5–8 per cent of serum immunoglobulins, with a normal level of 0.5–2 mg per ml. It has a half life of about five days. IgM molecules are polymers of five four-peptide subunits. With larger antigens, the effective valency falls to five, probably due to steric hindrance. Most of IgM (80 per cent) is intravascular in distribution. Phylogenetically, IgM is the oldest immunoglobulin class. It is also the earliest immunoglobulin to be synthesised by the fetus, beginning by about 20 weeks of age. As it is not transported across the placenta, the presence of IgM in the fetus or newborn indicates intrauterine infection and its detection is useful in the diagnosis of congenital infections such as syphilis, rubella, HIV infection and toxoplasmosis. IgM antibodies are relatively short lived, disappearing earlier than IgG. Hence, their demonstration in serum indicates recent infection.

The unique structural features of IgM appear particularly suited to the biological role of providing protection against microorganisms and other large antigens that have repeating antigenic determinants on their surface. Being largely confined to the intra-vascular space, IgM is believed to be responsible for protection against *blood invasion by microorganisms*. Monomeric IgM is the major antibody receptor on the surface of B-lymphocytes for antigen recognition (see figure 62).

IgE: It is a molecule with a half life of about two days. It resembles IgG structurally. It exhibits unique properties such as heat lability and affinity for the surface of tissue cells (particularly mast cells) of the same species. It does not pass the placental barrier or fix complement. It is mostly extravascular in distribution. Normal serum contains only traces (a few nanograms per ml) but greatly elevated levels are seen in atopic (type 1 allergy) conditions such as asthma, hay fever and eczema. Children living in insanitary conditions, with a high load of intestinal parasites, have high serum levels of IgE.

IgE is responsible for the *anaphylactic type of hypersensitivity* (type 1 allergy). The physiological role of IgE appears to be protection against pathogens by mast cell degranulation and release of inflammatory mediators. It is also believed to have a special role in defence against *helminthic infections*. In general, IgG protects the body fluids, IgA the body surfaces and IgM the bloodstream, while IgE mediates reaginic hypersensitivity. IgD is a recognition molecule on the surface of B-lymphocytes.

Table 21 — Classification of immunoglobulins

Property	Ig G	Ig A	Ig M	Ig E	Ig D
Serum concentration (mg/ml)	0.5–12	0.5–3	1.5	0.003–0.00003	0.03
Percentage of total immunoglobulins in serum	80	13	6	Less than 1	Less than 1
Half life (days)	23	6	5	1–5	2–8
Molecule weight	150.000	160.000	900.000	190.000	180.000
Complement fixation	Yes	No	Yes	No	No
Placental transport	Yes	No	No	No	No
Present in milk	Yes	Yes	No	No	No
Secretion by seromucous glands	No	Yes	No	No	NO
Number of subunits	Monomer	Dimer	Pentamer	Monomer	Monomer

IgD: IgD resembles IgG structurally. It is present in a concentration of about 3 mg per 100 ml of serum and is mostly intravascular. It has a half life of about three days. IgD and IgM occur on the surface of unstimulated B-lymphocytes and serve as *recognition receptors for antigens*. Combination of cell membrane bound IgD or IgM with the corresponding antigen leads to specific stimulation of the B-cells.

Structural differences between immunoglobulins

Isotype is determined by the primary sequence of amino acids in the constant region of the heavy chain. For example, the five human isotypes, IgA, IgD, IgG, IgE and IgM are found in all humans and injection of human IgG into another human would not generate antibodies directed against Ig G isotype. Another means of classifying immunoglobulins is defined by the term **allotype**. Unlike isotypes, allotypes reflect genetic differences between members of the same species. Therefore, injection of any specific human allotype into another human could possibly generate antibodies directed against particular allotypic variation. A third means of classifying immunoglobulins is defined by the term **idiotype** reflects the antigen binding specificity of any particular antibody molecule. Idiotypes are so unique that an individual person is probably capable of generating antibodies directed against their own idiotypic determinants.

Functions of antibodies in host defense

1. **Opsonization:** Phagocytosis is greatly enhanced when it is coated by antibodies.
2. **Steric hindrance**: Antibodies combine with the surfaces of microorganisms and may prevent their attachment to susceptible cells or mucosal surfaces.
3. **Toxin neutralization**: Toxin-neutralizing antibodies (**antitoxins**) react with a soluble bacterial toxin and block the interaction of the toxin with its specific target cell or substrate.
4. **Agglutination and precipitation**: Antibodies combine with microorganisms and cause them to agglutinate or precipitate. This reduces the number of separate infectious units and makes them more readily phagocytosed because the clump of particles is larger in size.
5. **Complement fixation**. Activation of the classic pathway occurs as a result of complement components binding to the C_{H2} region of Ig.

6. **Allergy and anaphylaxis**: Ag-specific Ig E may bind to the receptors on mast cells and promote their degranulation leading to the signs and symptoms of allergy.

7. **Ab-dependent cell-mediated cytotoxicity (ADCC):** Antibodies stimulated by virus infection bind to the viral Ag expressed on the surface of the infected cells. The Fc-portion of Ig binds to the Fc-receptor bearing cells which are able to lyse the viral infected cells.

8. **Effect on microbial physiology**: Some antibodies inhibit the movement organisms by attaching to flagella. Some Ab inhibit the metabolism of microbes.

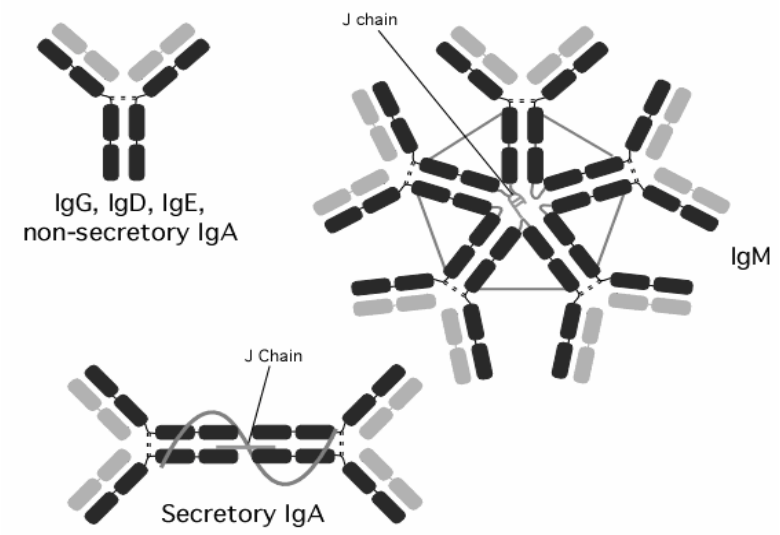


Figure 62 — Different classes of antibody

ANTIGEN-ANTIBODY REACTIONS

Non-covalent bonds: The bonds that hold the antigen to the antibody combining site are all non-covalent in nature. These include *hydrogen bonds, electrostatic bonds, Van der Waals forces* and *hydrophobic bonds*. Multiple bonding between the antigen and the antibody ensures that the antigen will be bound tightly to the antibody.

Reversibility: Since antigen-antibody reactions occur via non-covalent bonds, they are by their nature reversible.

Affinity and avidity

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 of the antibody.

Affinity is the equilibrium constant that describes the antigen-antibody reaction. Most antibodies have a high affinity for their antigens. **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.

To repeat, affinity refers to the strength of binding between a single antigenic determinant and an individual antibody combining site whereas avidity refers to the overall strength of binding between multivalent antigens and antibodies.

Specificity and cross reactivity

Specificity refers to the ability of an individual antibody combining site to react with only one antigenic determinant or the ability of a population of antibody molecules to react with only one antigen. In general, there is a high degree of specificity in antigen-antibody reactions. Antibodies can distinguish differences in 1) the primary structure of an antigen, 2) isomeric forms of an antigen, and 3) secondary and tertiary structure of an antigen.

Cross reactivity refers to the ability of an individual antibody combining site to react with more than one antigenic determinant or the ability of a population of antibody molecules to react with more than one antigen. Figure 63 illustrates how cross reactions can arise. Cross reactions arise because the cross reacting antigen shares an **epitope** in common with the immunizing antigen or because it has an epitope which is structurally similar to one on the immunizing antigen (**multispecificity**).

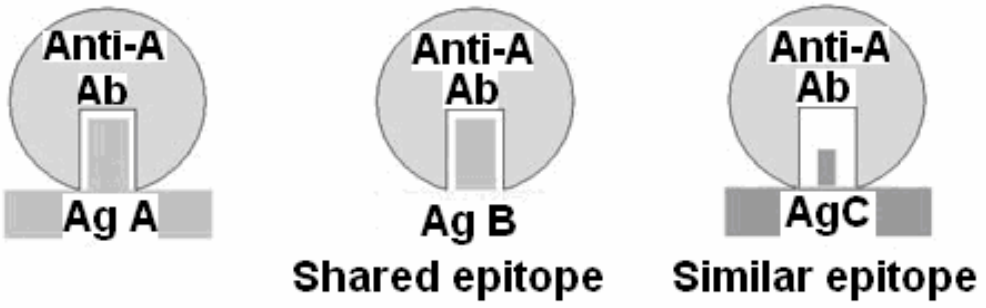


Figure 63 — Cross reactions

The reactions between antigens and antibodies serve several purposes. In the body, they form he *basis of antibody mediated immunity* in infectious diseases, or of tissue injury in some types of hypersensitivity and autoimmune diseases. In the laboratory, they help in the **diagnosis of infections**, in the identification of infectious agents and of noninfectious antigens such is enzymes. Antigen-antibody reactions in vitro are known as **serological reactions**.

The reactions between antigens and antibodies occur in two stages:

1. The **primary stage** is the *initial interaction without any visible effects. This reaction is rapid and reversible*. The primary reaction can be detected by estimating free and bound antigen or antibody separately in the reaction mixture by a number of physical and chemical methods, including the use of markers such as radioactive isotopes, fluorescent dyes.

2. The **secondary stage** is leading to demonstrable events such as *precipitation, agglutination, lysis of cells, killing of live antigens, neutralization of toxins and other biologically active antigens, fixation of complement, immobilization of motile organisms and enhancement of phagocytosis*. It was believed that a different type of antibody was responsible for each type of reaction and the antibodies came to be designated by the reactions they were produce.

Thus, the antibody causing agglutination was called *agglutinin,* that causing precipitation *precipitin,* and so on, and the corresponding antigen, *agglutinogen, precipitinogen,* and so on. The physical form of the antigen influences how one detects its reaction with an antibody. *If the antigen is a particulate*, one generally looks for agglutination of the antigen by the antibody*. If the antigen is soluble* one generally looks for the precipitation of the antigen after the production of large insoluble antigen-antibody complexes.

Precipitation reaction

When a **soluble antigen** combines with its antibody in the presence of electrolytes (NaCl) at a suitable temperature and pH, the antigen-antibody complex forms an **insoluble precipitate**. When, instead of sedimenting, the precipitate remains suspended as floccules, the reaction is known as **flocculation**. Precipitation can take place in liquid media or in gels such as agar. The amount of precipitate formed is greatly influenced by the relative proportions of antigens and antibodies. For a given antigen-antibody system, the optimal ratio will be constant, irrespective of the quantity of the reactants.

The following types of precipitation test are in common use: A. Ring test: the simplest type of precipitation test consists of layering the antigen solution over a column of antiserum in a narrow tube. Examples are **Ascoli's thermoprecipitin test** and the grouping of streptococci by the Lancefield technique (see figure 64).

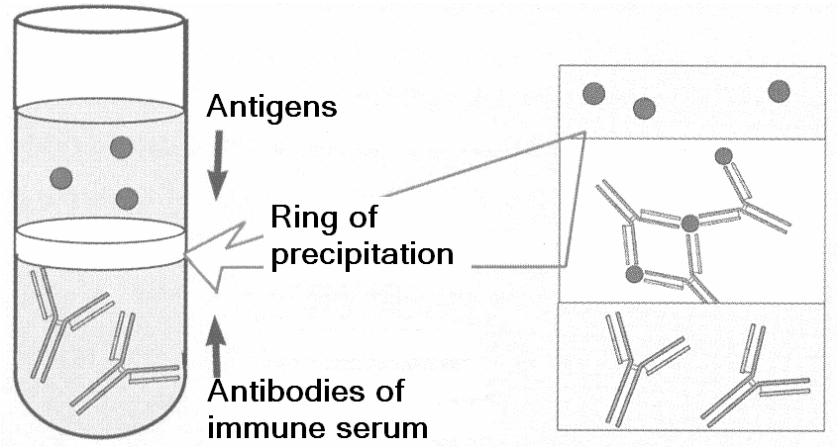


Figure 64 — Formation of precipitation ring

Slide and tube test: When a drop each of the antigen and antiserum are placed on a slide and mixed by shaking, floccules appear. The Kahn test for syphilis is an example of a tube flocculation test. Appearance flocculent sediment in the tube during of reaction toxin-antitoxin is employed for detection of **antitoxic serum (antitoxin) activity** (see figure 65).

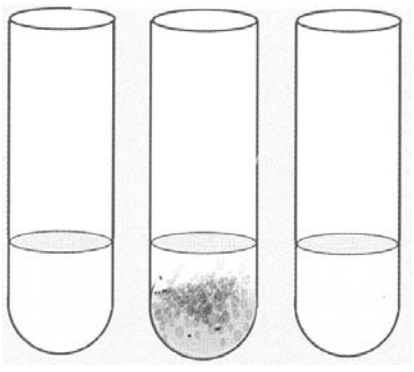


Figure 65 — Reaction of flocculation

Mechanism of precipitation: multivalent antigens combine with bivalent antibodies in varying proportions, depending on the antigen-antibody ratio in the reacting mixture. Precipitation results when a large lattice is formed consisting of alternating antigen and antibody molecules. This is possible only in the zone of equivalence. In the zones of antigen or antibody excess, the lattice does not enlarge, as the valencies of the antibody and the antigen, respectively, are fully satisfied. A precipitate forms at the junction of the two liquids. Ring tests have only a few clinical applications now.

Immunodiffusion (Precipitation in gel): The reaction is visible as a distinct band of precipitation, which is stable and can be stained for preservation, if necessary. As each antigen-antibody reaction gives rise to a line of precipitation, the number of different antigens in the reacting mixture can be readily observed.

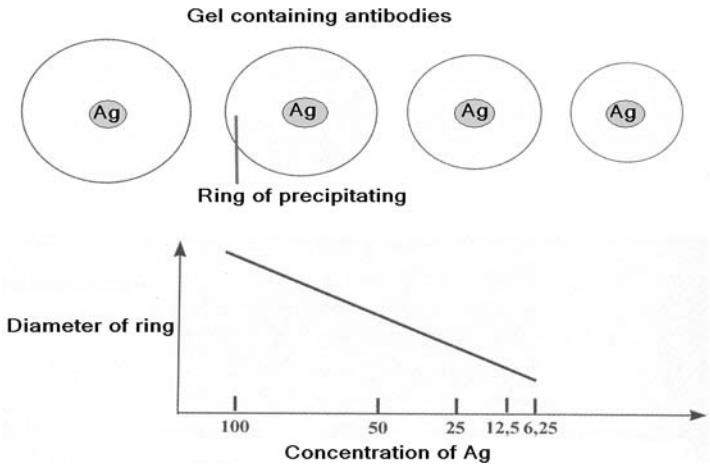


Figure 66 — Reaction of radial immunodiffusion

Different modifications of the precipitation test are available:

1. Single diffusion in one dimension*:* The antibody is incorporated in agar gel in a test tube and the antigen solution is layered over it. The antigen diffuses downward through the agar gel, forming a line of precipitation that appears to move downwards. This is due to the precipitation formed at the advancing front of the antigen, and is dissolved as the concentration of antigen at the site increases due to diffusion. The number of bands indicates the number of different antigens present.

2. Double diffusion in one dimension: Here, the antibody is incorporated in gel, above which is placed a column of plain agar. The antigen is layered on top of this. The antigen and antibody move towards each other through the intervening column of plain agar and form a band of precipitate where they meet at optimum proportion.

3. Single diffusion in two dimensions (Radial immunodiffusion): Antiserum is incorporated in agar gel poured on a flat surface. The antigen is added to the wells cut on the surface of the gel. It diffuses radially from the well and forms **ring** of precipitation concentrically around the well.

The diameter of the ring gives an estimate of the concentration of the antigen (see figure 66).

4. Double diffusion in two dimensions (Ouchterlony procedure):

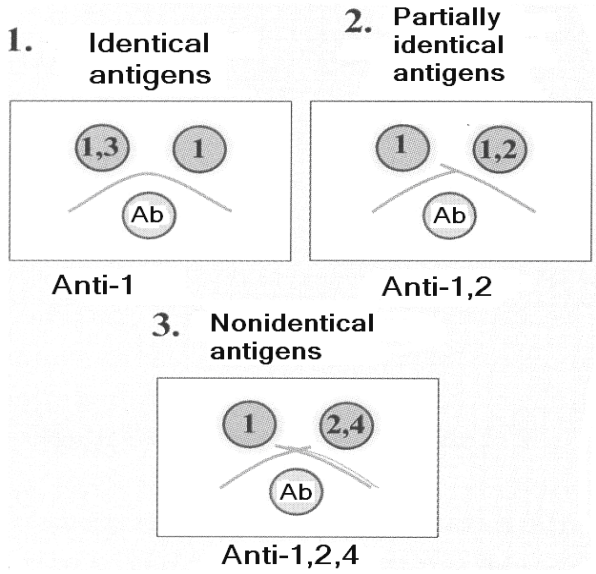


Figure 67 — Reaction of double diffusion

This is the immunodiffusion method most widely employed and helps to compare different antigens and antisera directly. Agar gel is poured on a slide and wells are cut using a template. The antiserum is placed in the central well and different antigens in the surrounding wells. If two antigens are identical, the lines of precipitate formed by them will fuse. If they are unrelated, the lines will cross each other (see figure 67).

The strains of *C. diphtheriae* may be **toxigenic** (producing exotoxins) and nontoxigenic. Formation of exotoxin is depended on a presence special tox-gene that is being encoding by prophage during **lysogenic conversion**. All isolated strains must be tested on toxigenicity with **reaction of precipitation in agar** during diphtheria (see figure 68).

Immunoelectrophoresis: This involves the electrophoretic separation of a composite antigen (such as serum) into its constituent proteins, followed by immunodiffusion against its antiserum, resulting in separate precipitin lines, indicating reaction between each individual protein with its antibody (see figure 69).

The test serum is placed in the antigen well and electrophoresed for about an hour. Antibody against human serum is then placed in the trough and diffusion allowed to proceed for 18–24 hours. The resulting precipitin lines can be photographed and the slides dried, stained and preserved for record. This is useful for testing for normal and abnormal proteins in serum and urine.

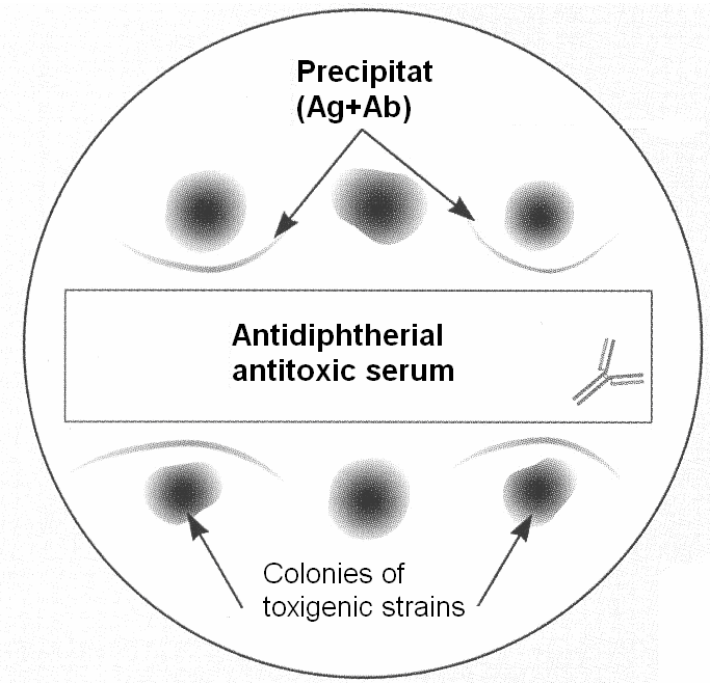


Figure 68 — Detection of toxigenicity of C. diphtheriae (precipitation in agar). There are nontoxigenic strains in the center.

Agglutination reaction

When a *particulate antigen* is mixed with its antibody in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated. The general term **agglutinin** is used to describe antibodies that agglutinate particulate antigens. Agglutination is more sensitive than precipitation for the detection of antibodies. The same principles govern agglutination and precipitation. *Agglutination occurs optimally when antigens and antibodies react in equivalent proportions*. All an-

tibodies can theoretically agglutinate particulate antigens but IgM, due to its high valence, is particularly good agglutinin and one sometimes infers that an antibody may be of the IgM class if it is a good agglutinating antibody.

The method of determining the presence of specific antigens in a microorganism is called **serological typing** (serotyping). It consists of adding a suspension of the organisms to **antiserum,** which contains antibodies that are specific for the known antigens. If the antigens are present, the antibodies in the antiserum will combine with the antigens, causing **agglutination,** or clumping, of the bacterial cells (see figure 70).

Serotyping is particularly useful in the identification of various organisms that cause salmonella and shigella infections. When the antigen is an erythrocyte the term **hemagglutination** is used.

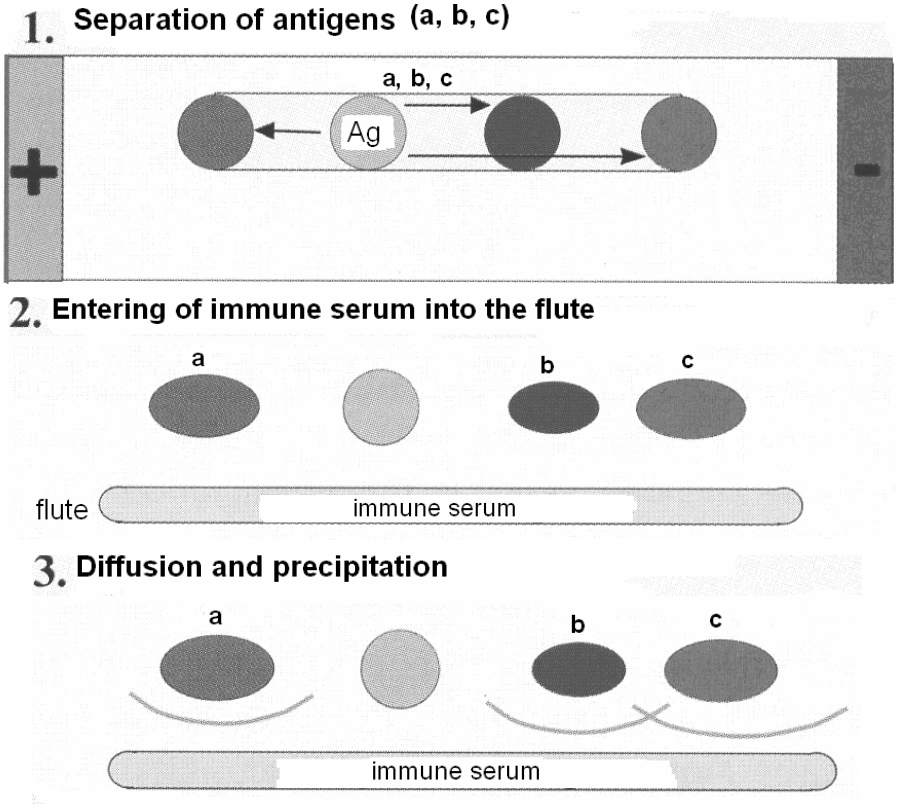


Figure 69 — Immunoelectrophoresis

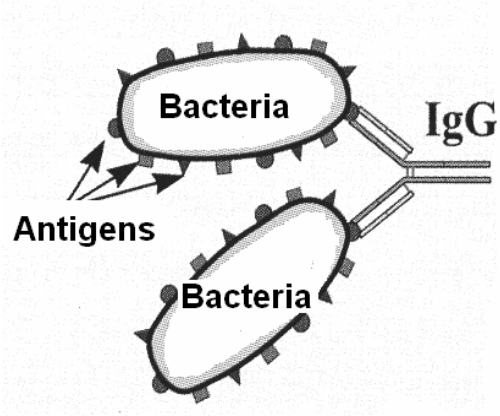


Figure 70 — Reaction of agglutination with Ig G

Detection of causative agent isolated from patient

Slide agglutination (tentative reaction or qualitative agglutination test): When a drop of the appropriate antiserum is added to a suspension of a particulate antigen in a drop of saline on a slide, agglutination takes place. A ***positive result*** is indicated by the clumping together of the particles and the clearing of the drop. The reaction is facilitated by mixing the antigen and the antiserum with a loop or by gently rocking the slide. Clumping occurring after a minute may be due to drying of the fluid and should be disregarded. It is essential to have on the same slide a control consisting of the antigen suspension in saline, without the antiserum, to ensure that the antigen is not autoagglutinable. Agglutination is usually visible to the naked eye but may sometimes require confirmation under the microscope (see figure 71).

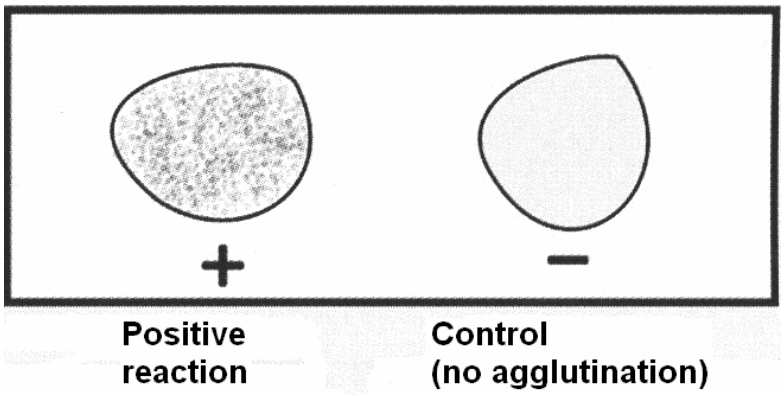


Figure 71 — Tentative reaction of agglutination on the glass

Slide agglutination is a routine procedure for *the identification of many bacterial isolates from clinical specimens*. For example, a patient's red blood cells can be mixed with antibody to a blood group antigen to determine a person's blood type. In a second example, a patient's serum is mixed with red blood cells of a known blood type to assay for the presence of antibodies to that blood type in the patient's serum.

Tube agglutination (detailed reaction or quantitative agglutination test): This is a standard quantitative method for the measurement of antibodies (see figure 72).

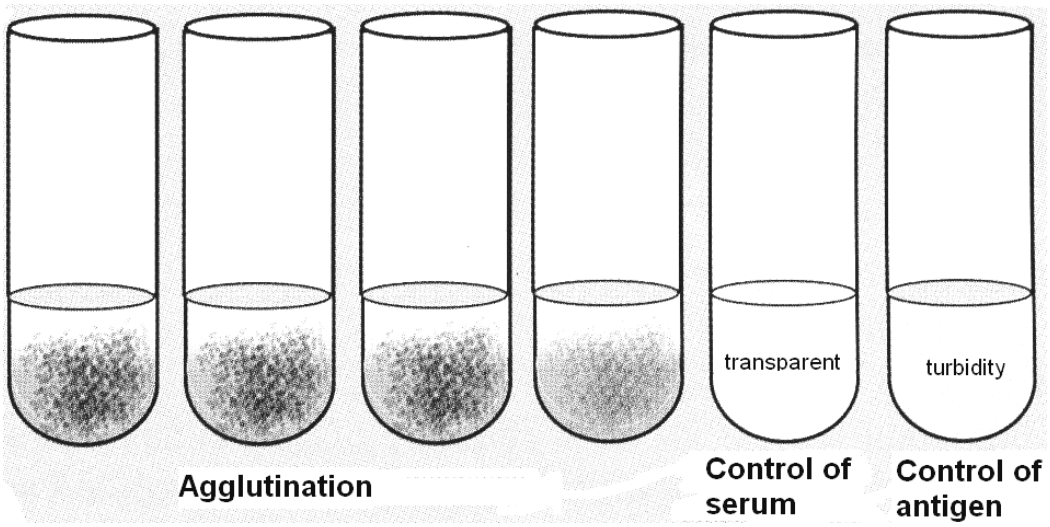


Figure 72 — Detailed reaction of agglutination

When a fixed volume of an antigen suspension is added to an equal volume of serial dilutions of an antiserum in test tubes, the agglutination titre of the serum can be estimated. Tube agglutination is routinely employed for the serological diagnosis *of typhoid, brucellosis and typhus fever*.

In the ***Widal test*** used in *typhoid*, two types of antigens are used. The “H” or the flagellar antigen on combining with its antibody forms large, loose, fluffy clumps resembling cotton wool. The “O” or somatic antigen forms tight, compact deposits resembling chalk powder. Agglutinated bacilli spread out in a disc like pattern at the bottom of the tubes.

The procedure involves adding a suspension of dead typhoid bacterial cells to a series of tubes containing the patient’s serum, which has been diluted out to various concentrations. After the tubes have been incubated for 30 minutes at 37° C, they are centrifuged and examined to note the amount of agglutination that has occurred.

The reciprocal of the highest dilution at which agglutination is seen is designated as the **antibody titer** of the patient’s serum. For example, if the highest dilution at which agglutination occurs is 1:320, the titer is 320 antibody units per milliliter of serum. Naturally, the higher the titer, the greater is the antibody response of the individual to the disease.

This technique can be used clinically to determine whether a patient with typhoid like symptomsactually has the disease. If successive daily tests on a patient’s serum reveal no antibody titer, or a low titer that does not increase from day to day, it can be assumed that some other disease is present. On the other hand, if one sees a daily increase in the titer, it can be assumed that a typhoid infection does exist.

Since the treatment of typhoid fever requires powerful antibiotics that are not widely used on other similar diseases, it is very important to diagnose this disease early to begin the proper form of chemotherapy as soon as possible.

When examining each tube, jar it first by rapping the side of the tube with a snap of the finger to suspend the clumps of agglutinated cells. Do not look directly into the light. The reflection of the light off the particles is best seen against a dark background.

Detection of antibodies in the patient serum (serodiagnosis)

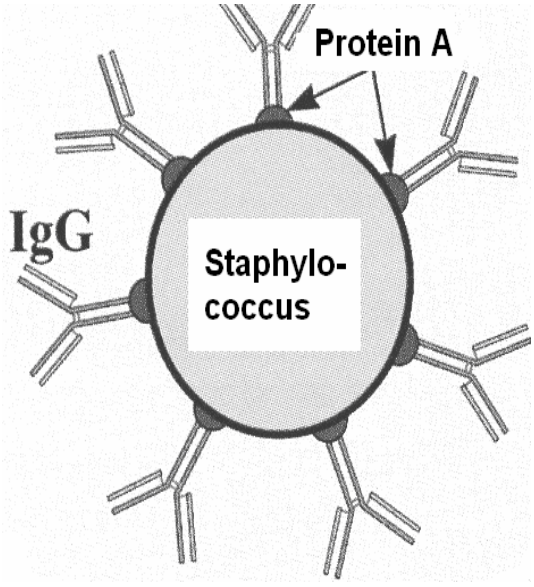


Figure 73 — Antibody-coated diagnosticum

Diagnosticums (see figure 73) are added *to serum dilutions of patient*. Agglutination with **O-diagnosticum** (bacteria are killed by heating and keeping O-Ag) results in formation of *microgranular agglutination*. Agglutination with **H-diagnosticum** (bacteria are killed by formalin and keeping H-Ag) results in formation of *macrogranular agglutination* and takes place faster (see figure 74).

The antiglobulin (Coombs) test: The antiglobulin test was devised by Coombs for the detection of anti-Rh antibodies that do not agglutinate Rh positive erythrocytes in saline. When sera containing incomplete anti-Rh antibodies are mixed with Rh positive red cells, the antibody globulin coats the surface of the erythrocytes, though they are not agglutinated.

When such erythrocytes coated with the antibody globulin are washed free of all unattached protein and treated with a rabbit antiserum against human gammaglobulin (antiglobulin or Coombs serum), the cells are agglutinated.

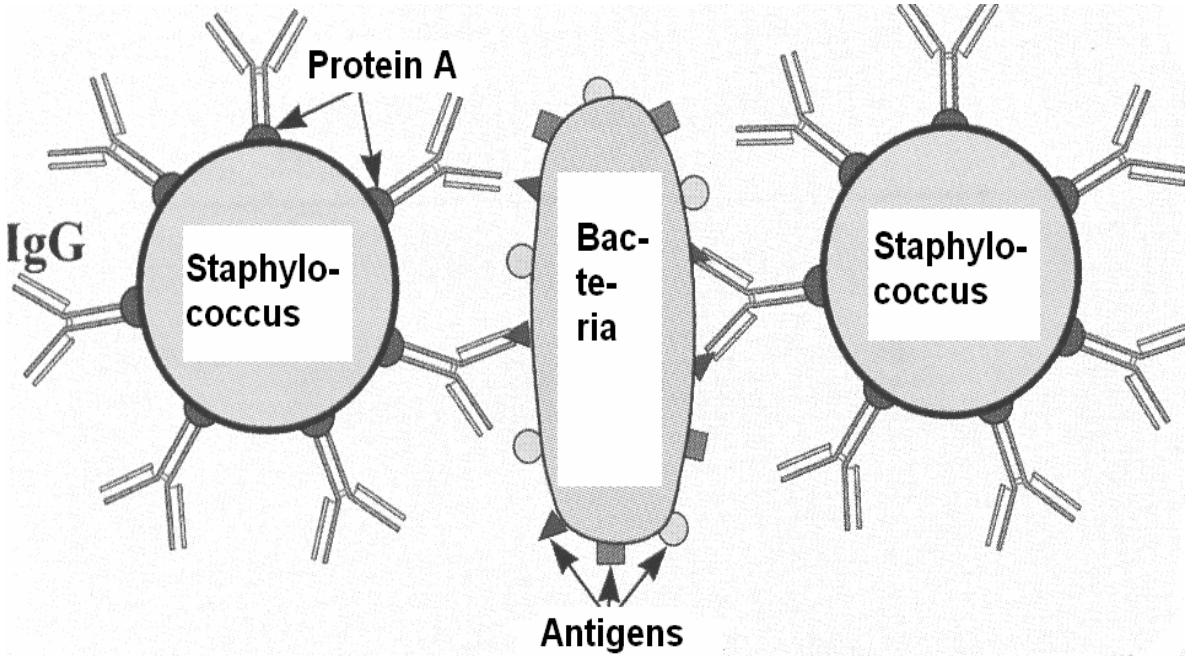


Figure 74 — Reaction of coagglutination

This is the principle of the antiglobulin test (Figure 75).

When antibodies bind to erythrocytes, they do not always result in agglutination. This can result from the antigen/antibody ratio being in antigen excess or antibody excess or in some cases electrical charges on the red blood cells preventing the effective cross linking of the cells. These antibodies that bind to but do not cause agglutination of red blood cells are sometimes referred to as incomplete antibodies. In no way is this meant to indicate that the antibodies are different in their structure, although this was once thought to be the case. Rather, it is a functional definition only. In order to detect the presence of non-agglutinating antibodies on red blood cells, one simply adds a second antibody directed against the immunoglobulin (antibody) coating the red cells. This anti-immunoglobulin can now cross link the red blood cells and result in agglutination. This test is illustrated in Figure 76 and is known as the **Direct Coomb's test**.

It is necessary to know whether a serum sample has antibodies directed against a particular red blood cell and you want to be sure that you also detect potential non-agglutinating antibodies in the sample, an **Indirect Coomb's test** is performed (Figure 77).

This test is done by incubating the red blood cells with the serum sample, washing out any unbound antibodies and then adding a second anti-immunoglobulin reagent to cross link the cells.

Applications: These include detection of **anti-rhesus factor (Rh)** antibodies. Antibodies to the Rh factor generally do not agglutinate red blood cells. Thus, red cells from Rh^+ children born to Rh^- mothers, who have anti-Rh antibodies, may be coated with these antibodies. To check for this, a direct Coombs test is performed. To see if the mother has anti-Rh antibodies in her serum an Indirect Coombs test is performed.

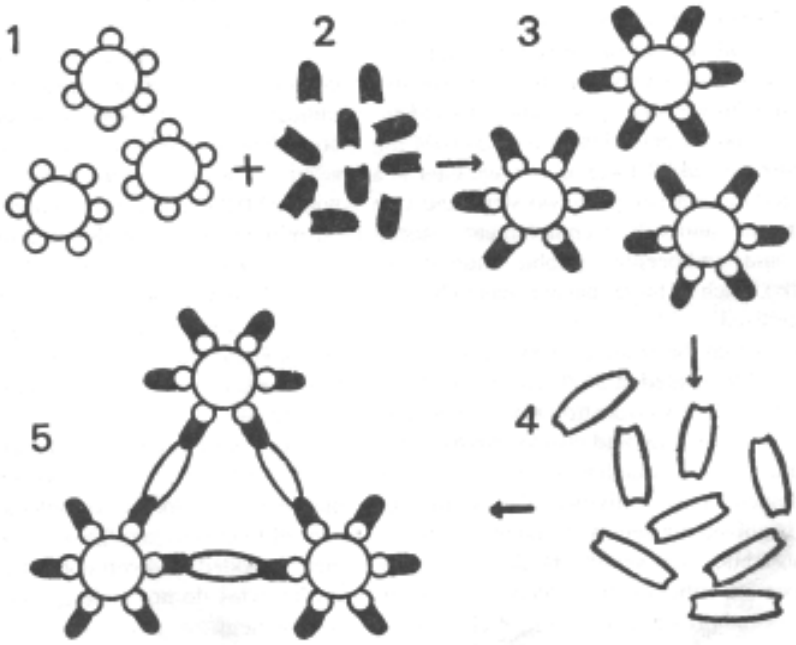


Figure 75 — Antiglobulin (Coombs) test.

Rh positive **erythrocytes** (1) are mixed with incomplete **antibody** (2). The antibody coats the **cells** (3) but, being incomplete, can not produce agglutination. On addition of **antiglobulin serum** (4) which is complete antibody to immnoglobulin, **agglutination** takes place (5).

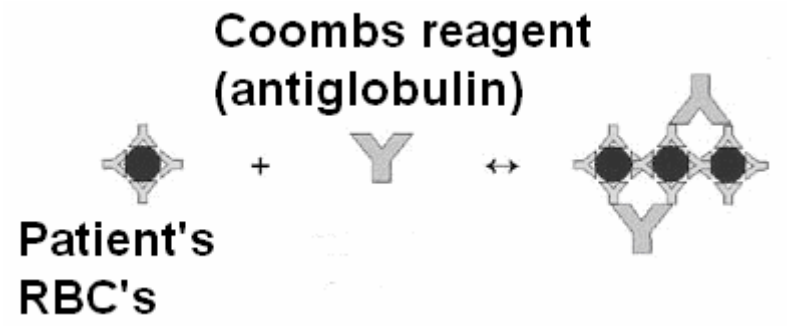


Figure 76 — Direct Coomb's test

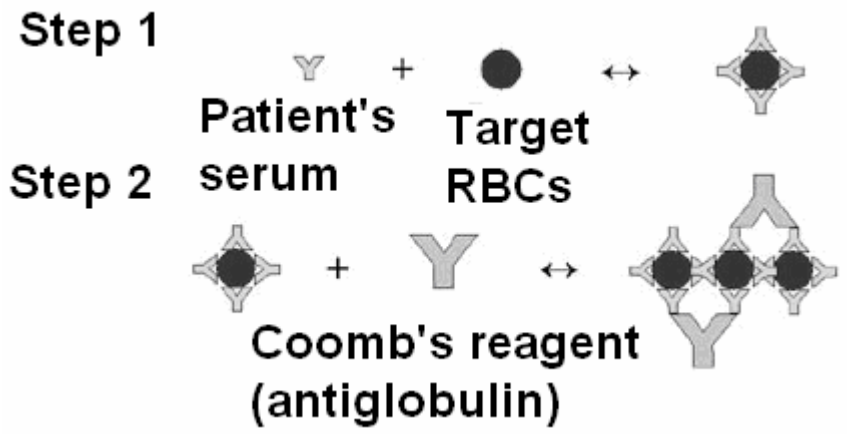


Figure 77 — Reaction of indirect Coombs test

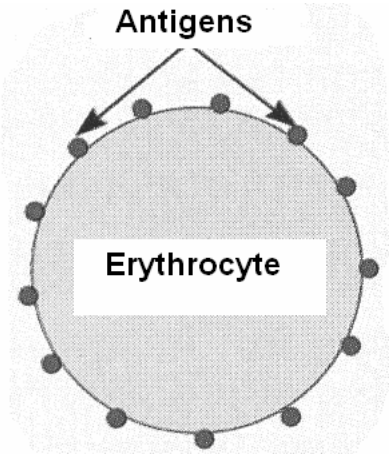


Figure 78 — Antigenic erithrocytic diagnosticum

Passive hemagglutination test: The only difference between the requirements for the precipitation and agglutination tests is the physical nature of the antigen. By attaching soluble antigens to the surface of carrier particles (cells, latex, etc), it is possible to convert precipitation tests into agglutination tests, which are more convenient and more sensitive for the detection of antibodies. Such tests are known as reaction **of passive agglutination tests.** It is possible to coat erythrocytes with a soluble antigen (e.g. viral antigen, a polysaccharide or a hapten) and use the coated red blood cells in an agglutination test for antibody to the soluble antigen (Figure 78). This is called **passive hemagglutination**.

Reaction of passive hemagglutination (RPHA) is employed for revealing of antibodies in the patient serum with *erithrocytic antigenic diagnosticum* (suspension of the erythrocytes with adsorbed superficial bacterial antigens). The erythrocytes with adsorbed Ag react with the corresponded antibodies of serum that cause sticking and deposition of scalloped sediment (like “umbrella”). Negative result includes the deposition of sediment like “button”.

Applications include detection of antibodies to soluble antigens and detection of antibodies to viral antigens.

It is employed an **erithrocytic diagnosticum coated by adsorbed antibodies** sometimes. Foe example, detection of the botulinic toxin is carried out by adding to it of *erithrocytic botulinic diagnosticum coated by antibodies*. It is called **reaction of reverse direct hemagglutination**.

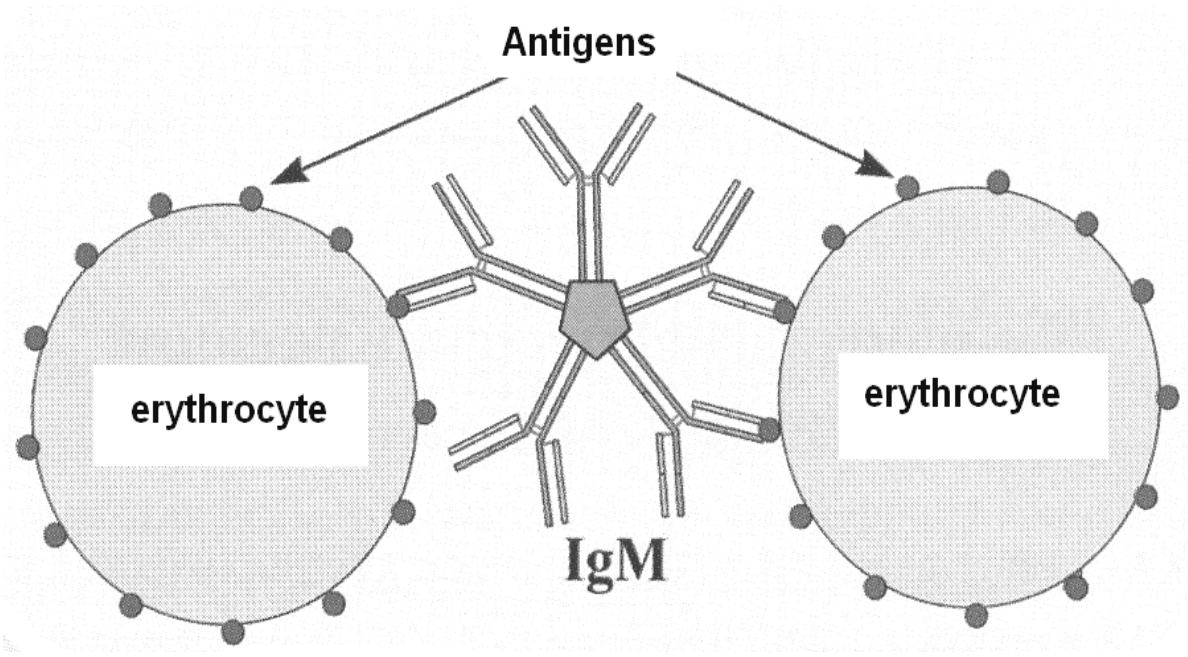


Figure 79 — Reaction of indirect hemagglutination

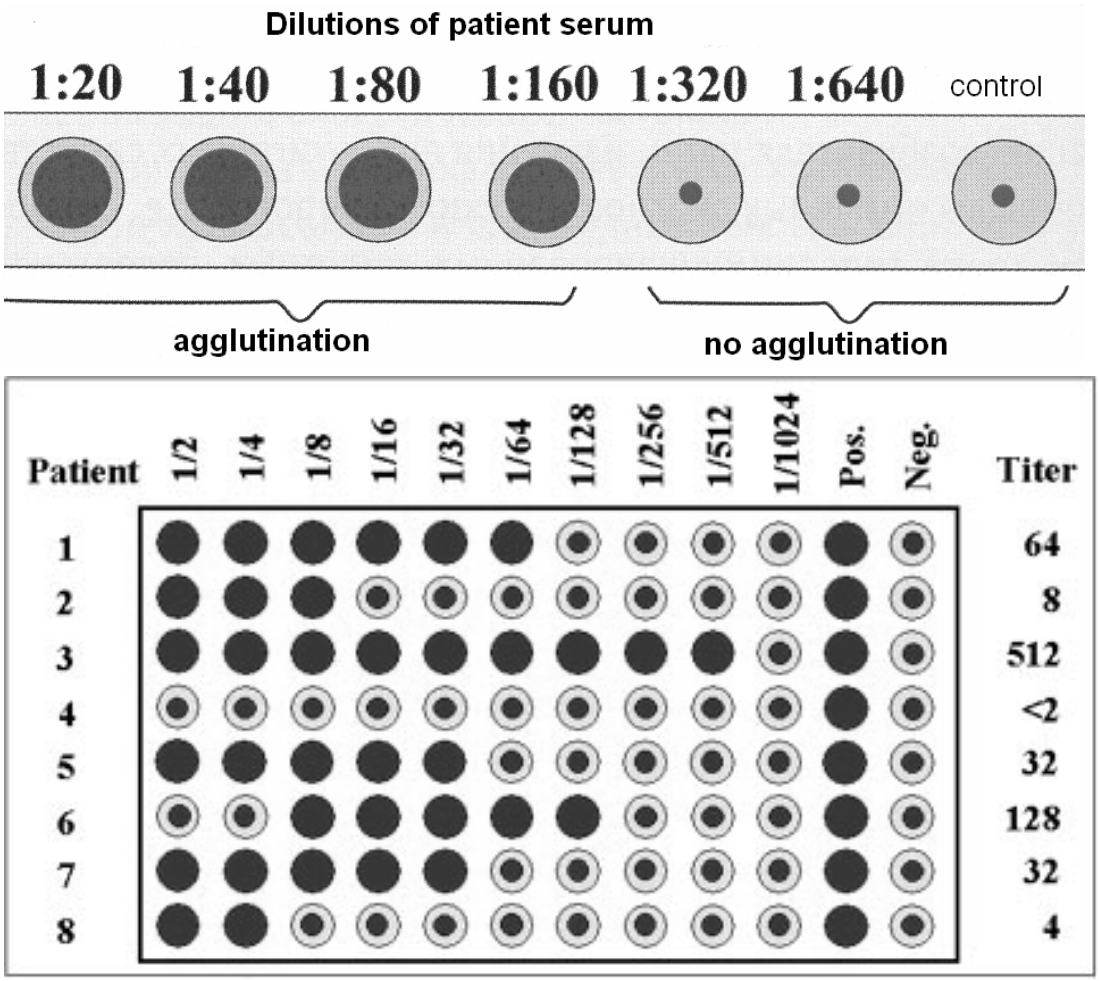


Figure 80 — Statement of RPHA

Applications of agglutination tests:

1. Determination of blood types or antibodies to blood group antigens.
2. To assess bacterial infections (*e.g.* a rise in titer of an antibody to a particular bacterium indicates an infection with that bacterial type. N.B. a fourfold rise in titer is generally taken as a significant rise in antibody titer).

Although the test is easy to perform, it is only semi-quantitative.

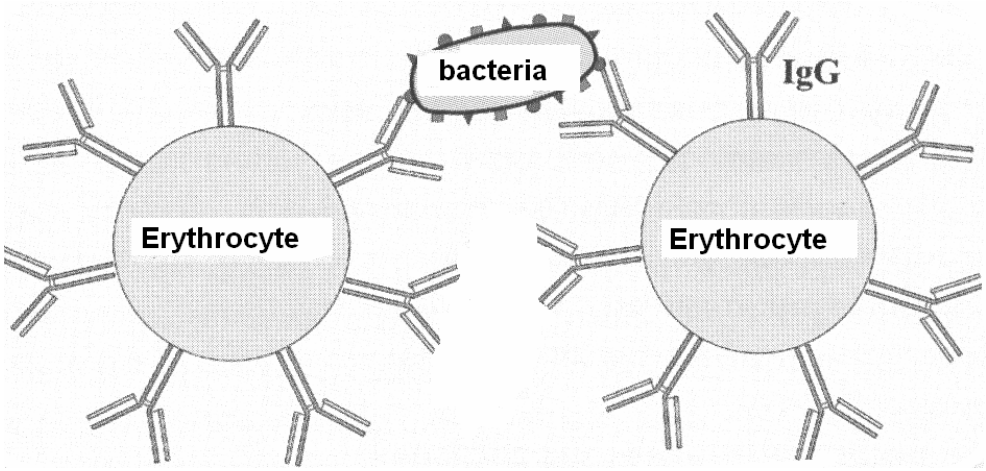


Figure 81 — Reaction of reverse direct hemagglutination

Hemagglutination inhibition

The agglutination test can be modified to be used for the measurement of soluble antigens. This test is called hemagglutination inhibition. It is called hemagglutination inhibition because one measures the ability of soluble antigen to inhibit the agglutination of antigen-coated red blood cells by antibodies. In this test, a fixed amount of antibodies to the antigen in question is mixed with a fixed amount of red blood cells coated with the antigen (see passive hemagglutination above).

Also included in the mixture are different amounts of the sample to be analyzed for the presence of the antigen. If the sample contains the antigen, the soluble antigen will compete with the antigen coated on the red blood cells for binding to the antibodies, thereby inhibiting the agglutination of the red blood cells as illustrated in Figure 82.

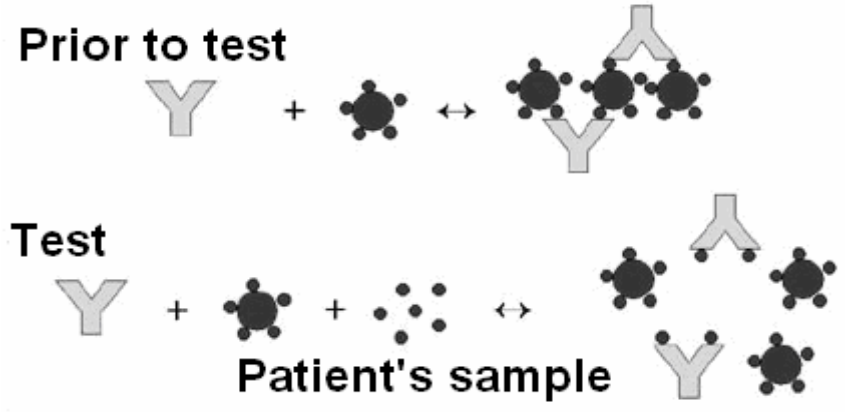


Figure 82 — Hemagglutination inhibition

By serially diluting the sample, you can quantitate the amount of antigen in your unknown sample by its titer. This test is generally used to quantitate soluble antigens and is subject to the same practical considerations as the agglutination test.

Neutralization tests

Virus neutralization tests: Neutralization of viruses by their antibodies can be demonstrated in various systems. Neutralization of bacteriophages can be demonstrated by the ***plaque inhibition test***. When bacteriophages are seeded in appropriate dilution on lawn cultures of susceptible bacteria, plaques of lysis are produced. Specific antiphage serum inhibits plaque formation.

Neutralization of animal viruses can be demonstrated in three systems — animals, eggs and tissue culture (see figure 83).

Toxin neutralization can be tested in vivo or in vitro.

Neutralization tests in animals consist of injecting toxin-antitoxin mixtures and estimating the least amount of antitoxin that prevents death or disease in the animals. With the diphtheria toxin, which in small doses causes a cutaneous (dermal) reaction, neutralization tests can be done on human skin. The **Schick test** is based on the ability of circulating antitoxin to neutralise the diphtheria toxin given intradermally, and indicates immunity or susceptibility to the disease. ***Toxin neutralization in vitro*** depends on the inhibition of some demonstrable toxic effect. An example is the antist-reptolysin O test, in which antitoxin present in patient's sera neutralizes the hemolytic activity of the streptococcal O hemolysin (see figure 84).

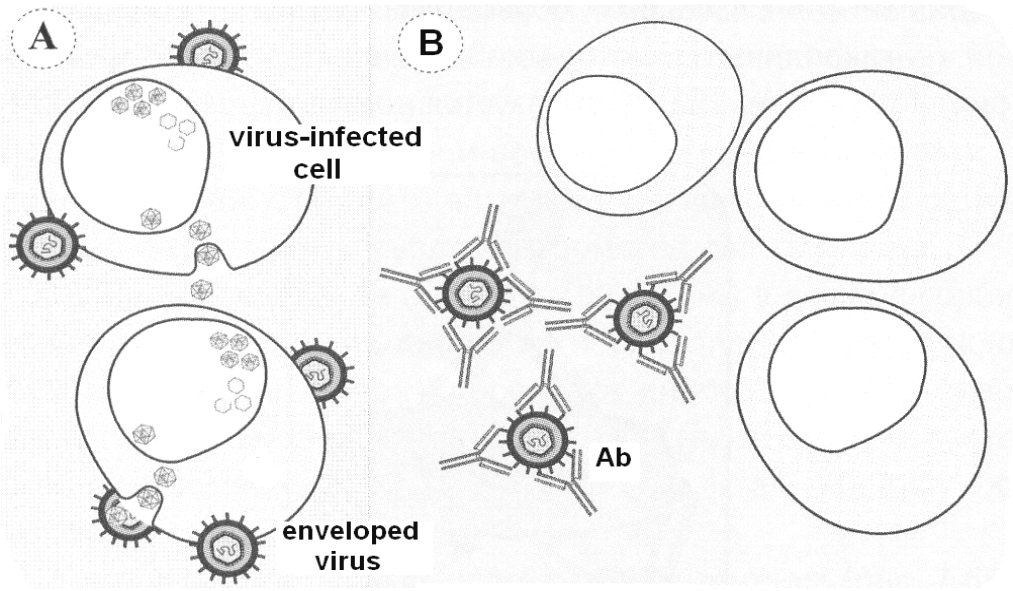


Figure 83 — Reaction of virus neutralization test in the tissue culture.
A — Cytopathogenic effect (CPE) in result of viral multiplication.
B – Viruses are inactivated by Ab and CPE is absent.

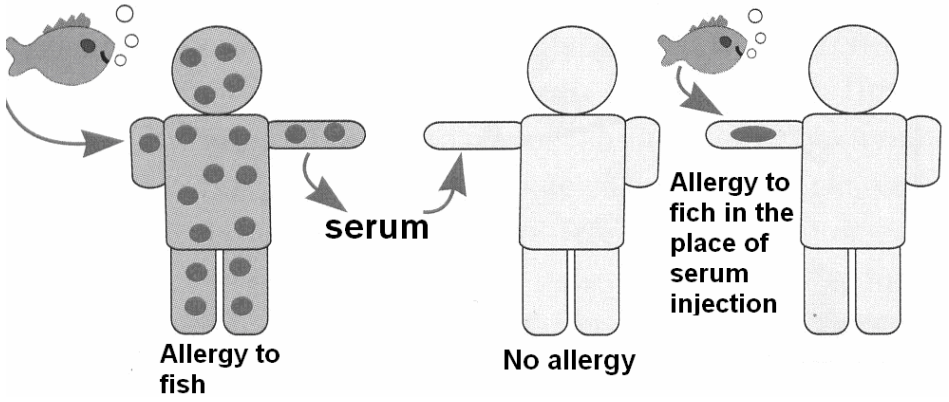


Figure 84 — Carry of allergy to fish with serum blood

Complement fixation test (CFT)

Complement takes part in many immunological reactions and is absorbed during the combination of antigens with their antibodies. The ability of **antigen antibody complexes** to 'fix' complement is made use of in the complement fixation test (CFT). The antigen may be soluble or particulate. The antiserum should be inactivated before the test to destroy any complement activity the serum may have and also to remove some nonspecific inhibitors of complement present in some sera (anticomplementary activity). The source of complement is *guinea pig serum*. The guinea pig serum should be titrated for complement activity. One unit or **minimum hemolytic dose** (MHD) of complement is defined as the highest dilution of the guinea pig serum that lyses one unit volume of washed sheep erythrocytes in the presence of excess hemolysin within a fixed time (usually 30 or 60 minutes) at a fixed temperature (37°C).

The classical example of CFT is the **Wassermann reaction**, formerly the routine method for the serodiagnosis of *syphilis*. The test consists of two steps.

In the first, the inactivated serum of the patient is incubated at 37°C for one hour with the Wassermann antigen and a fixed amount (two units) of guinea pig complement. If the serum contains syphilitic antibody the complement will be utilized during the antigen-antibody interaction. If the serum does not contain the antibody, no antigen-antibody reaction occurs and the complement will therefore be left intact. Testing for complement in the postincubation mixture will thus indicate whether the serum had antibodies or not (see figure 85).

This constitutes **the second step** in the test and consists of adding sensitized cells (sheep erythrocytes coated with 4 MHD hemolysin), and incubating at 37°C for 30 minutes. Lysis of the erythrocytes indicates that complement was not fixed in the first step and, therefore, the serum did not have the antibody (**negative CFT**). Absence of erythrocyte lysis indicates that the complement was used up in the first step and, therefore, the serum contained the antibody (**positive CFT**) as illustrated in figure 86.

Appropriate controls should be used, including the following: antigen and serum controls to ensure that they are not anticomplementary, complement control to ensure that the desired amount of complement is added, and cell control to see that sensitized erythrocytes do not undergo lysis in the absence of complement.

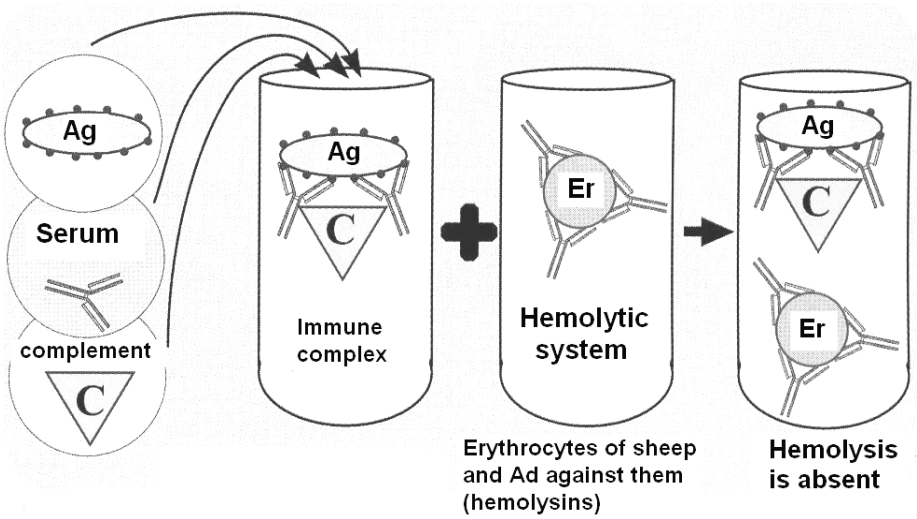


Figure 85 — Scheme of CFT with serum of sick person

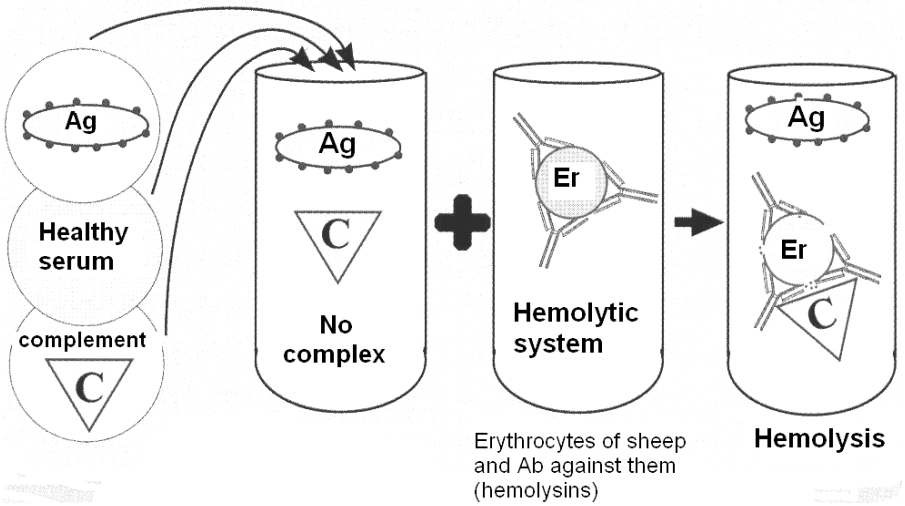


Figure 86 — Scheme of CFT with healthy serum

Other complement dependent serological tests: When some bacteria (for example, *Vibrio cholerae, Treponema pallidum)* react with the specific antibody in the presence of complement and particulate materials such as erythrocytes the bacteria are aggregated and adhere to the cells. This is known as ***immune adherence***. The ***immobilization test*** is another complement dependent reaction. In the *Treponema pallidum immobilization test,* a highly specific test formerly considered the "gold standard" for the serodiagnosis of syphilis, the test serum is mixed with a live motile suspension of *T. pallidum* in the presence of complement. On incubation, the specific antibody inhibits the motility of treponemes. ***Cytolytic* or *cytocidal tests*** are also complement dependent. When a suitable live bacterium, such as the cholera vibrio, is mixed with its antibody in the presence of complement, the bacterium is killed and lysed. This forms the basis of the vibriocidal antibody test for the measurement of anticholera antibodies.

Opsonization

The **"opsonin"** is heat labile substance present in fresh normal sera, which facilitated phagocytosis. The **opsonic index** was defined as the ratio of the phagocytic activity of the patient's blood for a given bacterium, to the phagocytic activity of blood from a normal individual. It was measured by incubating fresh citrated blood with the bacterial suspension at 37°C and estimating the average number of phagocytosed bacteria per polymorphonuclear leukocyte (phagocytic index) from stained blood films.

Immunofluorescence (tests for cell assocated antigens)

Fluorescence is the property of absorbing light rays of one particular wavelength and emitting rays with a different wavelength. Fluorescent dyes show up brightly under ultraviolet light as they convert ultraviolet into visible light. These fluorescent dyes can be conjugated to antibodies and that such *'labelled' antibodies* can be used to locate and identify antigens in tissues. This **"fluorescent antibody**" or **immunofluorescence technique** has several diagnostic and research applications. In its simplest forms (**direct immunofluorescence test**), it can be used for the identification of bacteria, viruses or other antigens, using the *specific antiserum labelled with a fluorescent dye*. For example, direct immunofluorescence is routinely used as a sensitive method of diagnosing rabies, by detection of the rabies virus antigens in brain smears (see figure 87).

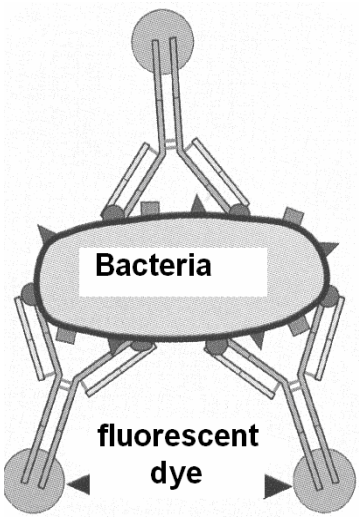


Figure 87 — Reaction of direct immunofluorescence

The "**indirect immunofluorescence test**" overcomes this difficulty by using an antiglobulin fluorescent conjugate. An example is the *fluorescent treponemal antibody test* for the diagnosis of *syphilis*. A drop of the test serum is placed on a smear of *T. pallidum* on a slide and after incubation, the slide is washed well to remove all free serum, leaving behind only antibody globulin, if present, coated on the surface of the treponemes. The smear is then treated with a *fluorescent labelled antiserum to human gamma-globulin*. The fluorescent conjugate reacts with antibody globulin bound to the treponemes. After washing away all the unbound fluorescent conjugate, when the slide is examined under ultraviolet illumination, if the **test is positive** the treponemes will be seen as bright objects against a dark background. If the serum does not have antitreponemal antibody, there will be no globulin coating on the treponemes and therefore they will not take on the fluorescent conjugates. A single antihuman globulin fluorescent conjugate can be employed for detecting human antibody to any antigen (Figure 88).

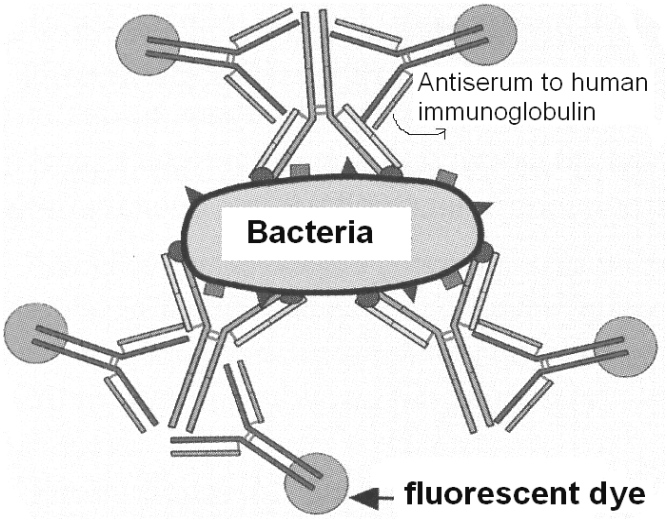


Figure 88 — Indirect immunofluorescence

Radioimmunoassay (RIA)

The most commonly used labels are **radioisotopes** and **enzymes**. A variety of tests have been devised for the measurement of antigens and antibodies using such labelled reactants. The term *binder-ligand-assay* has been used for these reactions. The substance (antigen) whose concentration is to be determined is termed the **ligand**. The binding protein (ordinarily, the antibody) which binds to the ligand is called the **binder**. RIA and its modifications have versatile applications in various areas of biology and medicine, including the quantitation of hormones, drugs, tumour markers, IgE and viral antigens.

RIA is a competitive binding assay in which fixed amounts of antibody and *radiolabelled antigen* react in the presence of unlabelled antigen. The labelled and unlabelled antigens compete for the limited binding sites on the antibody. This competition is determined by the level of the unlabelled (test) antigen present in the reacting system.

After the reaction, the antigen is separated into "free" and "bound": fractions (***labelled immune complex***) and their radioactive counts measured. The concentration of the test antigen can be calculated from the ratio of the bound and total antigen labels, using a standard dose response curve (*intensity of radiation is directly proportional to the amount of bound Ag and Ab*).

Enzyme immunoassay (EIA)

The term *enzyme immunoassay* (EIA) includes all assays based on the revealing of the **antigens** with corresponded them **antibodies** labelled with enzyme (**peroxidase** or **phosphatase**). **Substrate/chromogen** is added after combining of antigens with the labelled immune serum (antibodies). Substrate is decomposed by an enzyme and colour of reaction product is changing. *Intensity of colouring is directly proportional to the amount of bound Ag and Ab molecules.*

EIAs are of two basic types — homogeneous and heterogeneous. In **homogeneous EIA**, there is no need to separate the bound and free fractions so that the test can be completed in one step, with all reagents added simultaneously. This type of EIA can be used only for assay of haptens such as *drags* and not for microbial antigens and antibodies. An example of homogeneous EIA is ***enzyme multiplied immunoassay technique*** **(EMIT)**, which is a simple assay method for small molecule drugs such as opiates, cocaine, barbiturates or amphetamine in serum. ***Heterogeneous*** **EIA** requires the separation of the free and bound fractions either by centrifugation or by absorption on solid surfaces and washing. It is therefore a multistep procedure, with reagents added sequentially. The major type of heterogeneous EIA is **Enzyme Linked Immunosorbent Assay (ELISA)**.

ELISA is so named because the technique involves the use of an *immunosorbent* is an absorbing material specific for one of the components of the reaction, the antigen or antibody. This may be cellulose or agarose, polyacrylamide, paper or plastic surfaces (**solid-phase ELISA**). Components (Ag or Ab) are revealed by means of adding the labelled Ab or Ag respectively. ***Positive result is change of colour of the substrate/chromogen.*** Every time after of adding of the next component the unbound reagents are removed by washing.

1. Competitive RIA/ELISA for Ag detection

The method and principle of RIA and ELISA for the measurement of antigen is shown in Figure 89.

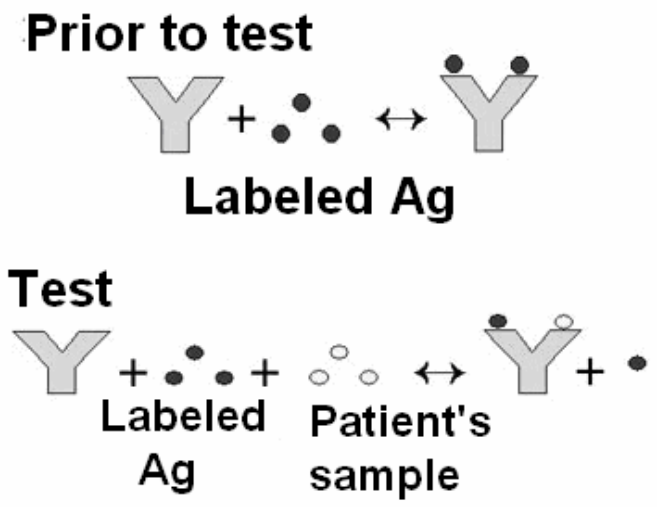


Figure 89 — Competitive RIA/ELISA for Ag detection

By using known amounts of a standard unlabeled antigen, one can generate a standard curve relating radioactivity (cpm) (Enzyme) bound versus amount of antigen. From this standard curve, one can determine the amount of an antigen in an unknown sample.

The key to the assay is the separation of the immune complexes from the remainder of the components. This has been accomplished in many different ways and serves as the basis for the names given to the assay:

a**. Precipitation with ammonium sulphate**

Ammonium sulphate (33–50% final concentration) will precipitate immunoglobulins but not many antigens. Thus, this can be used to separate the immune complexes from free antigen. This has been called the **Farr Technique.**

b. **Anti-immunoglobulin antibody**

The addition of a second antibody directed against the first antibody can result in the precipitation of the immune complexes and thus the separation of the complexes from free antigen.

c**. Immobilization of the Antibody**

The antibody can be immobilized onto the surface of a plastic bead or coated onto the surface of a plastic plate and thus the immune complexes can easily be separated from the other components by simply washing the beads or plate (Figure 90). This is the most common method used today and is referred to as solid phase RIA or ELISA. In the clinical laboratory, competitive RIA and ELISA are commonly used to quantitate serum proteins, hormones, drugs metabolites.

2. Noncompetitive RIA/ELISA for Ag or Ab

Noncompetitive RIA and ELISAs are also used for the measurement of antigens and antibodies. In Figure 91, the bead is coated with the antigen and is used for the detection of antibody in the unknown sample.

The amount of labeled second antibody bound is related to the amount of antibody in the unknown sample. This assay is commonly employed for the measurement of antibodies of the IgE class directed against particular allergens by using a known allergen as antigen and anti-IgE antibodies as the labeled reagent. It is called the RAST test (radioallergosorbent test).

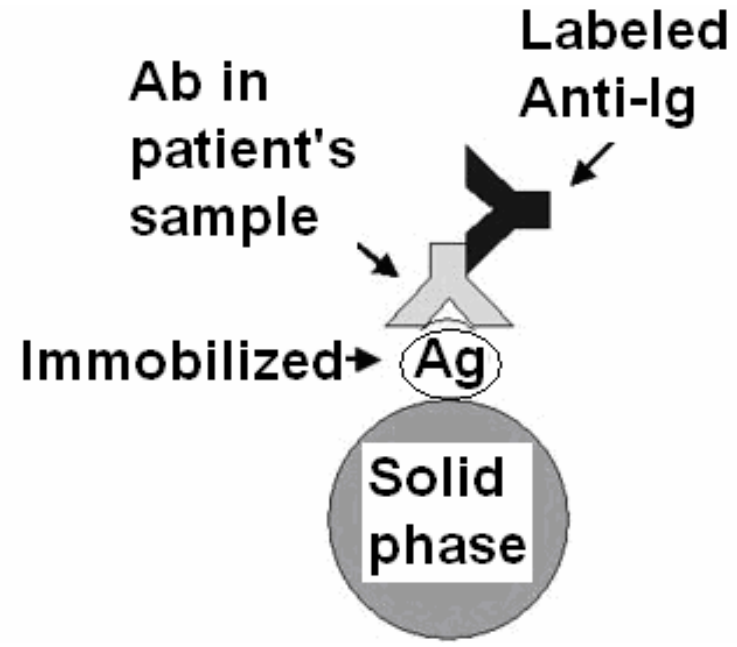


Figure 90 — Immobilization of Ab

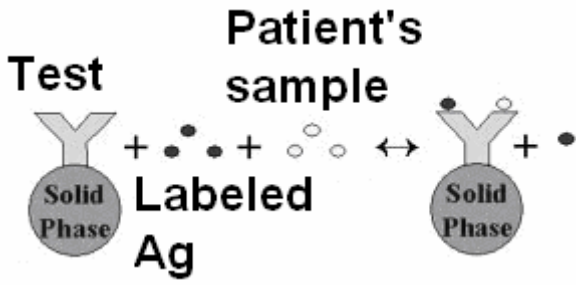


Figure 91 — Noncompetitive RIA/ELISA for Ag or Ab

In Figure 92, the bead is coated with antibody and is used to measure an unknown antigen. The amount of labeled second antibody that binds is proportional to the amount of antigen that bound to the first antibody.

1. During of **detection of antibodies** (figure) there are adsorbed antigens onto solid surface; then the *serum of sick person (with human Ig)*, serum against human Ig labelled with enzyme (*antiglobulin serum*) and *substrate/chromogen* for the enzyme are added consistently (see figure 93).

2. During of **detection of antigens** (figure) there are adsorbed antibodies onto solid surface; then the *antigen* (example, blood serum of sick person with required antigen), diagnostic serum against given antigen and secondary antibodies (against diagnostic serum) labelled with the enzyme and then substrate/chromogen for the enzyme are added consistently (see figure 94).

Also the detection of antibody by ELISA can be illustrated by the *anti-HIV antibody test*. Purified inactivated HIV-antigen is adsorbed onto microassay plate wells. Test serum diluted in buffer is added to the well and incubated at 37°C for 30 minutes. The well is then completely washed. If the serum contains anti-HIV antibody, it will form a stable complex with the HIV-antigen on the plate. A *goat antihuman immunoglobulin antibody* conjugated with horse radish peroxidase enzyme is added and incubated for 30 minutes. After complete washing, the *substrate O-phenylene diamine dihydrochloride* is added and after 30 minutes, *the* colour that develops is read using a microassay plate reader. Positive and negative controls should invariably be used with test serum.

EIA is employed for diagnostics of the viral, bacterial and parasitic infections and for detection of the enzymes, hormones and drugs containing in the observed materials the minor amount (10^{10} – 10^{12} g/l).

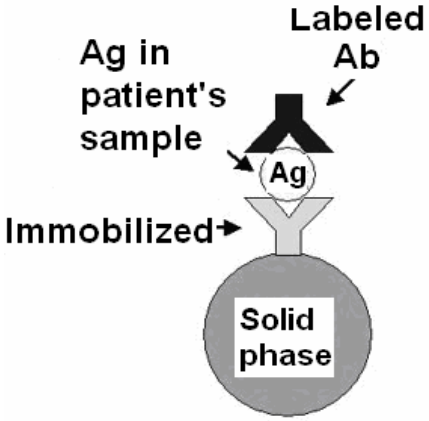


Figure 92 — Bead is coated with antibody

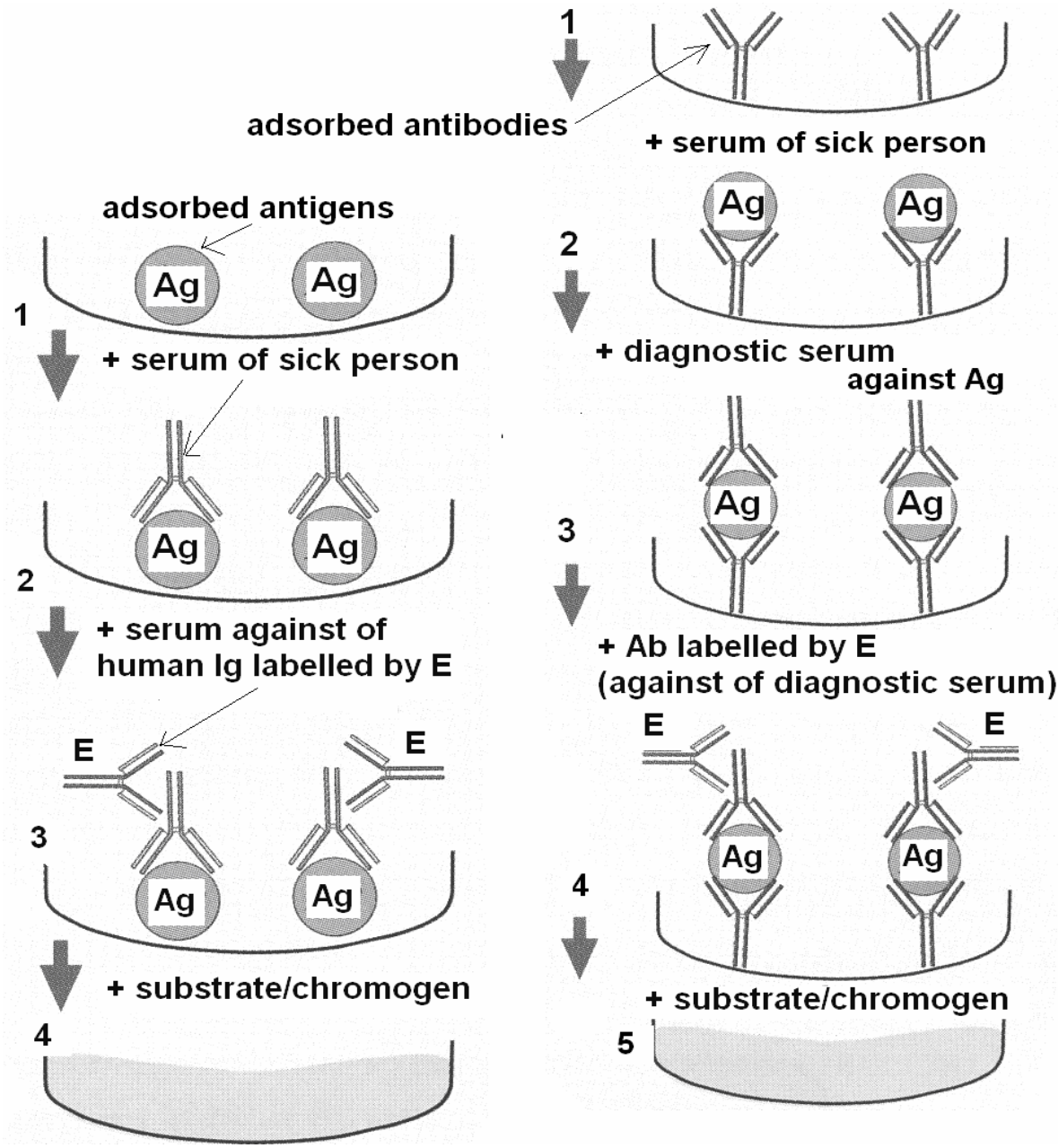


Figure 93 — Detection of Ab in the blood

Figure 94 — Detection of Ag in the blood

Immunoelectroblot techniques

Immunoelectroblot techniques combine the sensitivity of enzyme immunoassay with much greater specificity. The technique is a combination of several separate procedures (figure): **separation** of ligand-antigen components by ***polyacrylamide gel electrophoresis***, **blotting** of the electrophoresed ligand fraction on *nitrocellulose membrane strips or activated paper* **(1)** and display of Ag with enzyme immunoassay **(EIA)** or radio immunoassay **(RIA)**. The firms produce such papers and strips with antigenic blots. Serum of sick person is covered the paper **(2)**. After incubation the unbound antibodies are washed out and paper is covered by serum against human antibodies (anti-Ig-serum) labeled with enzyme **(3)**. Formed on the paper immune complex (Ag-Ab-anti-Ig-serum) is revealed by means of adding substrate/chromogen **(4)** that can change the colour under the influence of enzyme (see figure 95).

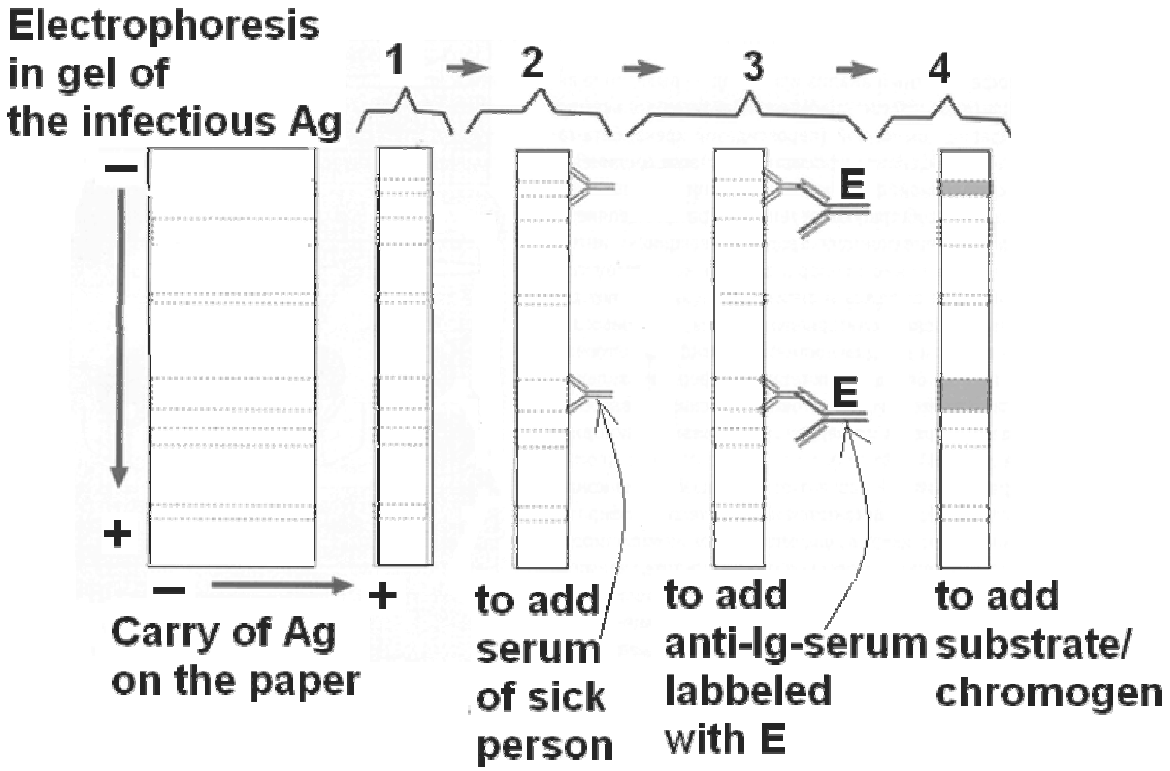


Figure 95 — Immunoelectroblotting

Immunoelectroblotting is employed for diagnostics of HIV-infection and other.

STRUCTURE AND FUNCTIONS OF THE IMMUNE SYSTEM

The immune system consists of factors that provide innate and acquired immunity, and has evolved to become more specific, complex, efficient, and regulated. *One of the principal functions of the human immune system is to defend against infecting and other foreign agents by distinguishing self from non-self (foreign antigens) and to marshal other protective responses from leukocytes.* The immune system, if dysregulated, can react to self antigens to cause **autoimmune diseases** or fail to defend against infections.

The immune system is organized into **discrete compartments** to provide the development and maintenance of effective immunity: the **lymphoid** and **reticuloendothelial systems** (RES) house the principal immunologic cells, the **leukocytes**.

Leukocytes derived from pluripotent stem cells in the bone marrow during postnatal life include **neutrophils**, **eosinophils**, **basophils**, **monocytes** and **macrophages**, **natural killer (NK) cells**, and **T** and **B-lymphocytes**.

Cells of the immune system intercommunicate by ligand-receptor interactions between cells and/or via secreted molecules called **cytokines**. Cytokines produced by lymphocytes are termed lymphokines (i.e., interleukins and interferon-gamma) and those produced by monocytes and macrophages are termed monokines.

Three important features of the immunological system relevant to host defense and/or "immunity" to pathogenic microorganisms are:

1. **Specificity**. An antibody or reactive T-cell will react specifically with the antigen that induced its formation; it will not react with other antigens. Generally, this specificity is of the same order as that of enzyme-substrate specificity or receptor-

ligand specificity. However, cross-reactivity is possible. The specificity of the immune response is explained on the basis of the clonal selection hypothesis: during the primary immune response, a specific antigen selects a pre-existing clone of specific lymphocytes and stimulates exclusively its activation, proliferation and differentiation.

2. **Memory**. The immunological system has a "memory". Once the immunological response has reacted to produce a specific type of antibody or reactive T cell, it is capable of producing more of the antibody or activated T cell more rapidly and in larger amounts. This is sometimes referred to as a **secondary,** or **memory response**.

3. **Tolerance**. An animal generally does not undergo an immunological response to its own (potentially-antigenic) components. The animal is said to be **tolerant**, or unable to react to its own potentially-antigenic components. This ensures that under normal conditions, an immune response to "self" antigens (called an **autoimmune response**) does not occur. Autoimmune responses are potentially harmful to the host. Tolerance is brought about in a number of ways, but basically the immunological system is able to distinguish "self" components from "non-self" (foreign) antigens; it will respond to "non-self" but not to "self". Sometimes in an animal, tolerance can be "broken", which may result in an autoimmune disease.

Organization of immune system:

1. The **lymphoid system** consists of the **lymphoreticular cells** and **lymphoid organs**.

2. The **lymphoreticular system** is a complex organization of cells of diverse morphology distributed widely in different organs and tissues of the body. Lymphoreticular cells consist of **lymphoid** and **myeloid** (**reticuloendothelial)** components.

3. The **lymphoid cells** (*lymphocytes and plasma cells*) are primarily concerned with the *specific immune response*. The *phagocytic cells*, forming part of the **reticuloendothelial system**, are primarily concerned with the functions of eliminating effete cells and foreign particles. They contribute to *nonspecific immunity* by removing microorganisms from blood and tissues.

4. Based on the different roles they perform, **lymphoid organs** can be classified into the **central** (primary) and the **peripheral** (secondary) lymphoid organs.

The **functional anatomy** of the lymphoid system can be appreciated only against the background of the “two component concept” of immunity. The immunological system is able to recognize foreign substances (antigens) which stimulate the system to produce **humoral or antibody-mediated immunity (AMI)** and **cell-mediated immunity (CMI)**. This process is illustrated in figure 96.

Antigen is a substance, usually macromolecular, that induces an immunological response. Single microorganism consists of multiple antigens (e.g. surface structures such as cell wall components, fimbriae, flagella, toxins, enzymes produced by the microorganism). The coat proteins and some of the envelope proteins of animal viruses are also usually antigenic. The host is able to respond specifically to every antigen that comes into contact with the immunological system. Lymphocytes responsible for AMI are processed by lymphoid tissue in the bone marrow and develop there into **B-lymphocytes** or **B-cells)**. Lymphocytes responsible for CMI are processed by the thymus gland and mature into **T-lymphocytes** or **T-cells**. Under antigenic stimulus, B-lymphocytes become transformed into antibody-secreting **plasma cells**. The plasma cells synthesize large amounts of **immunoglobulins** (**antibodies**) which will react with the stimulating antigen. The secreted antibody binds to the antigen and in some way leads to its neutralization or elimination from the body.

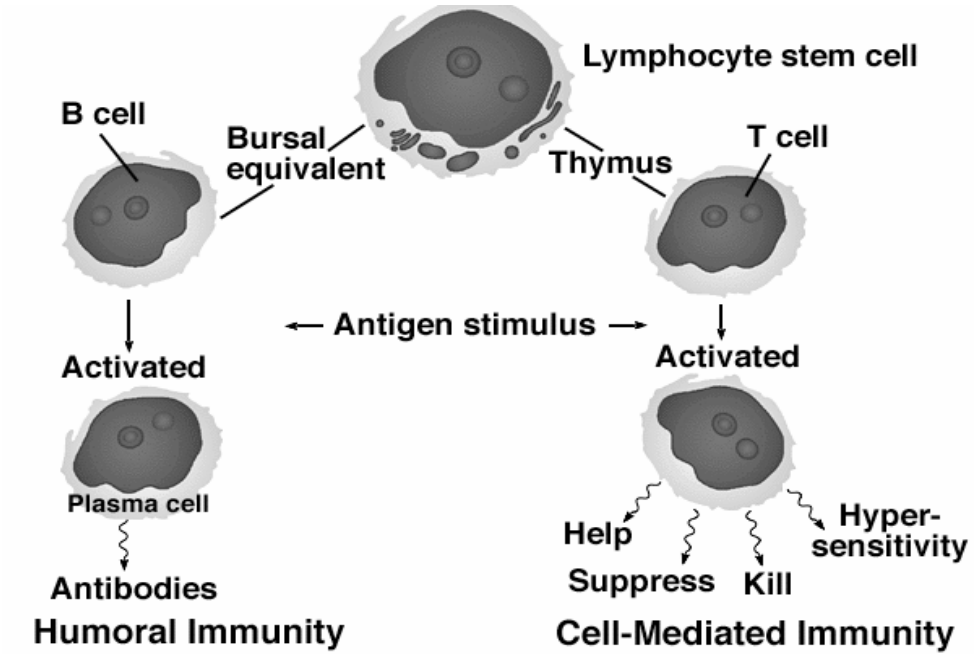


Figure 96 — Development of T- and B-system

A number of surface antigens or markers have been identified on lymphocytes and other leucocytes by means of monoclonal antibodies. These markers reflect the stage of differentiation and functional properties of the cells. Order was introduced at the 'International Workshops for Leucocyte Differentiation Antigens' by comparing the specificities of different antisera. When a cluster of monoclonal antibodies was found to react with a particular antigen, it was defined as a separate marker and given a CD (**cluster of differentiation**) number. Over 150 CD markers have been identified so far.

Contrasting roles of the AMI and CMI responses in host defense:

Antibody mediated immunity provides primary defence against most extracellular bacterial pathogens, helps in defence against viruses that infect through the respiratory or intestinal tracts (poliomyelitis or yellow fever), prevents recurrence of virus infections and participates in the pathogenesis of immediate (types 1, 2 and 3) hypersensitivity and certain autoimmune diseases, diseases caused by circulating bacterial toxins (e.g. diphtheria and tetanus).

Cell mediated immunity protects against fungi, viruses (e.g. herpes, pox viruses and measles virus infections) and facultative intracellular bacterial pathogens, provides immunological surveillance and immunity against cancer, and mediates the pathogenesis of delayed (type 4) hypersensitivity and certain autoimmune diseases, in rejection of tissue transplants in animals.

The central and peripheral lymphoid organs

The **central lymphoid organs** are lymphoepithelial structures in which the precursor lymphocytes proliferate, develop and acquire immunological capability. The **thymus** and the **bursa of Fabricius in birds** are primary lymphoid organs, being responsible for the cellular and humoral immune responses, respectively. The equivalent of the bursa in mammals is **bone marrow**.

After acquiring immunocompetence, the lymphocytes migrate along blood and lymph streams, accumulate in the **peripheral lymphoid organs** and, following antigenic stimu-

lus, effect the appropriate immune response. The **spleen**, **lymph nodes** and **mucosa-associated lymphoid tissue** constitute the major peripheral lymphoid organs. Lymphoid tissue in the gut, lungs, liver and bone marrow and lymphoid collections in the adventitious tissue of all organs also form part of the peripheral lymphoid system (see figure 97).

Thymus: In the thymus gland lymphoid cells undergo a process of maturation and education prior to release into the circulation. This process allows T-cells to develop the important attribute known as self tolerance.

Anatomy: The thymus gland is found in the thorax in the anterior mediastinum. It gradually enlarges during childhood but after puberty it undergoes a process of involution resulting in a reduction in the functioning mass of the gland. It continues to function throughout life.

Histology: The thymus gland is arranged into an outer, more cellular, cortex and an inner, less cellular, medulla. Immature lymphoid cells enter the cortex proliferate, mature and pass on to the medulla. From the medulla mature T lymphocytes enter the circulation. T-lymphocytes are selectively seeded into certain sites in the peripheral lymphatic tissues, being found in the white pulp of the spleen, around the central arterioles, and in the paracortical areas of lymph nodes. These regions have been termed **'thymus dependent'** as they are found grossly depleted after neonatal thymectomy. While thymectomy affects CMI primarily, it also diminishes antibody response to many types of antigens (**thymus dependent antigens**) such as sheep erythrocytes.

Approximately three-quarters of all the lymphocytes in the thymus are located in the deeper cortex. These cells express CD1 and both CD4 and CD8 (T-cells in the blood express either CD4 or CD8). Cells, which express antigen receptors that readily recognise molecules usually present in the body (selfantigens) in association with products of the MHC, are induced to die by a process called apoptosis (non-necrotic cell death which prevents the release of intracellular molecules). If such self-reactive cells were released to the periphery, they might cause damage to normal body cells (autoimmune T-cells). Indeed, the majority of cells produced in the thymus die there.

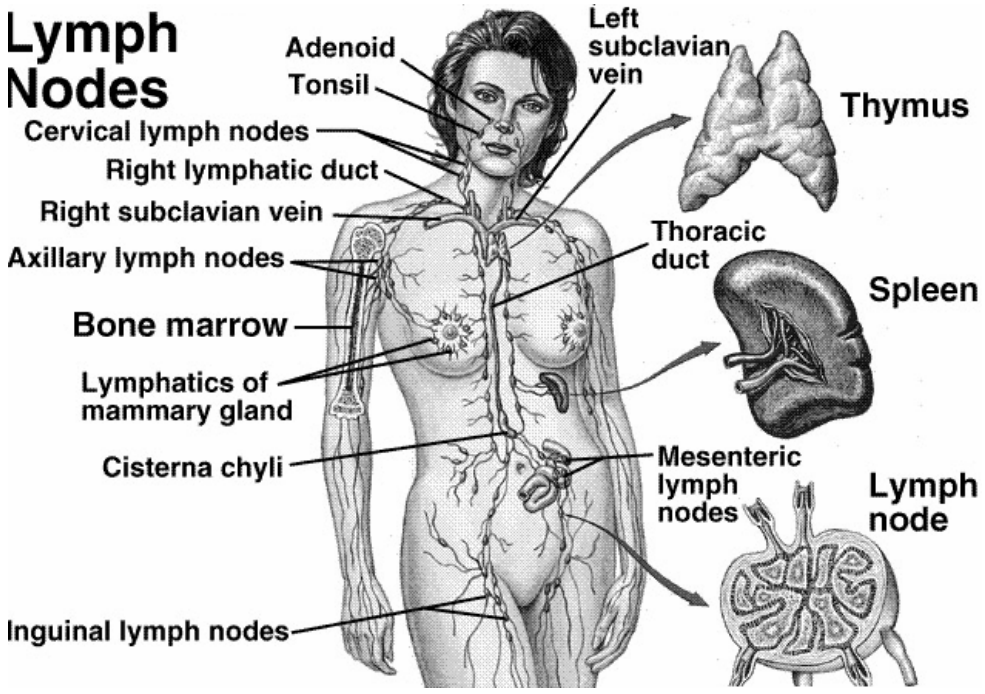


Figure 97 — Lymphoid system

All the cells of the immune system are derived from stem cells in the **bone marrow**. The bone marrow is the site of origin of red blood cells, white cells (including lymphocytes and macrophages) and platelets (thrombocyte).

The **bursa of Fabricius** in birds is a lymphoepithelial organ located near the cloaca. Birds which have this organ removed are not able to mount a normal antibody response when stimulated with an antigen. Thus, the bursa is a primary lymphoid tissue concerned with the development and differentiation of B-cells. In mammals, those tissues that most closely resemble the bursa include the gut-associated lymphoid tissues (GALT; including the appendix and Peyer's patches), the foetal liver and, following birth, the bone marrow.

All lymphocytes develop initially from haemopoietic stem cells in the bone marrow. Unlike precursor T-cells, which migrate to the thymus for further development, immature B-cells remain in the bone marrow and develop into mature cells under local influences.

The immature B-cells interact with the bone marrow stromal cells that provide signals for the precursor cells to undergo a number of defined developmental steps. These processes are influenced by a series of cell surface ligands and cytokines.

Lymph nodes: Lymph nodes are placed along the course of lymphatic vessels. They are surrounded by a fibrous capsule from which penetrate into the nodes. The node can be differentiated into an outer cortex and an inner medulla. In the cortex are accumulations of lymphocytes (*primary lymphoid follicles*) within which germinal centers (*secondary follicles*) develop during antigenic stimulation. The follicles contain, besides proliferating lymphocytes, dendritic macrophages which capture and process the antigen. The cortical follicles contain B-lymphocytes. Paracortical area contains T-lymphocytes.

Lymph nodes act as a filter for the lymph, each group of nodes draining a specific part of the body. They phagocytosed foreign materials including microorganisms. They help in the proliferation and circulation of T and B-cells. They enlarge following local antigenic stimulation.

Spleen: The spleen is the largest of the lymphoid organs. It has a capsule from which descend, dividing the organ into several interconnected compartments. The spleen serves as the graveyard for effete blood cells, as a reserve tank and settling bed for blood and as a systemic filter for trapping circulating bloodborne foreign particles. The immunological function of the spleen is primarily directed against bloodborne antigens.

Mucosa associated lymphoid tissue (MALT): These areas are provided with a rich collection of lymphoid cells, either specialized aggregates like the Peyer's patches or scattered isolated lymphoid follicles — collectively called the mucosa associated lymphoid tissue (MALT). Such lymphoid tissues in the gut, from the adenoids and tonsils to the follicles in the colon, are called the **gut associated lymphoid tissue** (GALT) and those in the respiratory tract, the **bronchus associated lymphoid tissue** (BALT).

MALT contains lymphoid as well as phagocytic cells. Both B and T-cells are present. While the predominant immunoglobulin produced in the mucosa is secretory IgA, other immunoglobulin classes, IgG, IgM and IgE are also formed locally.

Cells of the lymphoreticular system

Leukocytes, the main cells in the immune system, provide either innate or specific adaptive immunity. These cells are derived from (1) **myeloid** or (2) **lymphoid** lineage.

● **Myeloid cells** include highly phagocytic, motile **neutrophils**, **monocytes**, and **macrophages** that provide a *first line of defense against most pathogens*. The other myeloid cells, including **eosinophils**, **basophils**, and their tissue counterparts, **mast cells**, are involved in defense *against parasites* and in the genesis of *allergic reactions*. In contrast, lymphocytes regulate the action of other leukocytes and generate specific immune responses that prevent chronic or recurrent infections.

***Neutrophils**:* These are one of the major types of cells that are recruited to ingest, kill, and digest pathogens. Neutrophils are the most highly adherent, motile, phagocytic leukocytes and are the first cells recruited to acute inflammatory sites. Each of their functions is dependent upon special proteins, such as the adherence molecule CD11b/CD18.

***Eosinophils**:* Eosinophils defend against many types of parasites and participate in common hypersensitivity reactions via cytotoxicity. That cytotoxicity is mediated by large cytoplasmic granules, which contain the eosinophilic basic and cationic proteins.

***Basophils**:* These cells and their tissue counterparts, mast cells, produce **cytokines** that help defend against parasites and engender allergic inflammation. These cells display high affinity surface membrane receptors for IgE antibodies and have many large cytoplasmic granules, which contain heparin and histamine. When cell-bound IgE antibodies are cross-linked by antigens, the cells degranulate and produce mediators (e.g. histamine) through which they exert their biological effects (see figure 98).

***Monocytes/Macrophages**:* Monocytes and macrophages are involved in phagocytosis and intracellular killing of microorganisms. Macrophages process protein antigens and present peptides to T-cells. These monocytes/macrophages are highly adherent, motile and phagocytic; they regulate other cells of the immune system, such as T-lymphocytes; serve as **antigen processing-presenting cells**; and act as cytotoxic cells when armed with specific IgG antibodies.

Macrophages are differentiated **monocytes**, which are one of the principal cells found to reside for long periods in the myeloid system. Macrophages may also be recruited to inflammatory sites, and be further activated by exposure to certain cytokines to become more effective in their biologic functions.

Mononuclear macrophage cells originate in the bone marrow from precursor cells and become monocytes in about six days. Monocytes in circulation have an approximate half life of three days. They leave the circulation and reach various tissues to become transformed into macrophages, with morphological and functional features characteristic of the tissues, such as alveolar macrophages in the lungs and Kupffer cells in the liver. Tissue macrophages proliferate locally and survive for months.

The primary function of macrophages is **phagocytosis**. These cells move slowly and accumulate in areas of inflammation or tissue damage by chemotaxis. Macrophages express many surface receptors including Ia proteins, those for the *Fc* part of IgG, activated complement components and various lymphokines.

The *processing and presentation of antigen by the macrophage* to Th-cells require that both the cells possess surface determinants *coded for by the same major histocompatibility complex (MHC) genes*. The Th-cell can accept the processed antigen only if it is presented by a macrophage carrying on its surface the self-MHC antigens. When the macrophage bears a different MHC antigen, it cannot cooperate with the T-cells (*MHC restriction).*

Dendritic cells: While macrophages are the major *antigen presenting cells,* another type of cell known as the *dendritic cell* also performs this function. Dendritic cells are bone marrow derived cells of a lineage different from the macrophages and T or B-lymphocytes. They possess MHC Class II antigens but not *Fc*-receptors. They have little or no phagocytic activity. They are present in the peripheral blood and in the peripheral lymphoid organs, particularly in the germinal areas of the spleen and lymph nodes. Dendritic cells are especially involved in the presentation of antigens to T-cells during the *primary immune response*.

● **Lymphoid cells** provide efficient, specific and long-lasting immunity against microbes and are responsible for acquired immunity. Lymphocytes comprise about 20% of the peripheral white blood cells and derive from the common lymphoid progenitor cell in the bone marrow. They are about 6–10 mm in diameter and have a large, almost spherical nucleus surrounded by a very small, indistinct halo of cytoplasm. Their most important characteristic is their ability to specifically recognise foreign (non-self) molecules such as microorganisms, a feature not possessed by any other cell. This means the function of any lymphocyte stimulated by a foreign molecule, or antigen, is directed solely at that antigen and usually no other. Upon stimulation, some lymphocytes become **effector cells** performing functions designed to eliminate the antigen whilst others form long-lived memory cells that may persist for years and allow a more rapid response upon subsequent exposure to the antigen.

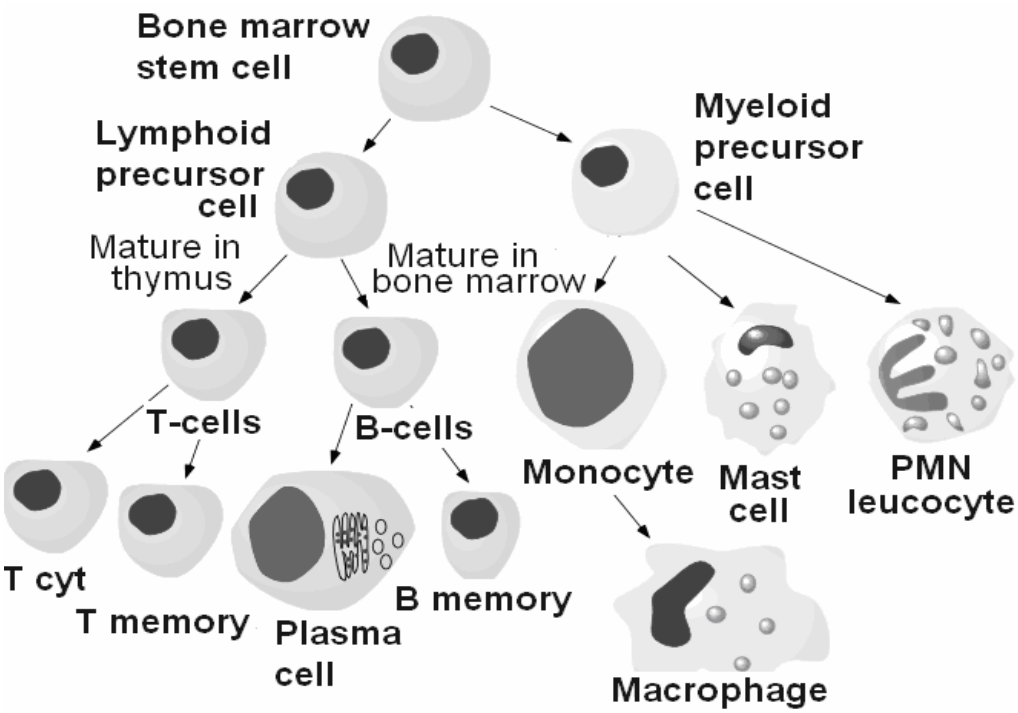


Figure 98 — Blood cell development

There are two major populations of lymphocytes – **T-cells** and **B-cells**. Although they are derived from a common progenitor in the bone marrow, they are ‘‘conditioned’’ or ‘‘educated’’ by the Thymus or the Bone marrow (respectively) before they become functionally active. Lymphocytes that have been 'educated' by the central lymphoid organs become an **'immunologically competent cells'** (ICC). Mature T and B-cells, before they encounter antigens are called **naive cells**. Such cells, though not actually engaged in an immunological response, are nevertheless fully qualified to

undertake such a responsibility when appropriately stimulated by an antigen. They subserve the following functions — recognition of antigens, storage of immunological memory, and immune response to specific antigens.

These lymphocyte populations may be distinguished by the molecules they express on their surface membrane and by the substances they secrete. **T (and other) cells** produce **lymphokines**. By contrast, only **B-cells** produce **antibodies**. T and B-cell populations are distinguished by the presence or absence of particular molecules found in their cell membranes (see figure 99).

Table 22 — Characteristics that distinguish T from B-lymphocytes

Characteristic	T-cells	B-cells
Cell type	Mononuclear leukocyte / lymphocyte	Mononuclear leukocyte / lymphocyte
Membrane molecules that allow binding of Ag – the Ag-receptor	T-cell Ag-receptor (TcR) and CD3	Superficial Ig (Ig D and IgM), CD 79a/b, B-cell Ag-receptor (BcR)
Characteristic surface membrane molecules	CD3, CD4 or CD8	Membrane Ig, CD 19, CD20, CD 40
Chief secretory products	Lymphokines	Antibodies

Table 23 — Immunocompetent cells

Cell group	Surface components	Function
B-lymphocytes	• Surface immunoglobulins (IgD, IgM) • Fc-receptor • Class II Major Histocompatability Complex (MHC)	• Direct antigen recognition • Differentiation into antibody-producing plasma cells • Antigen presentation within Class II MHC
T-lymphocytes	• CD3 molecule • T-cell receptor (TcR)	• Involved in both humoral and cell-mediated responses
Helper T-cells	• CD4 molecule	• Promotes differentiation of B-cells and cytotoxic T-cells • Activates macrophages
Suppressor T-cells	• CD8 molecule	• Downregulates the activities of other cells
Cytotoxic T-cells (T killer)	• CD8 molecule	• Recognizes antigen presented within Class I MHC • Kills cells expressing appropriate antigen
Accessory cells (myeloid cells)	•Variable	•Phagocytosis and cell killing
Macrophages	• Fc-receptor • Complement component C3b receptor • Class II MHC molecule	• Bind Fc portion of immunoglobulin (enhances phagocytosis) • Bind complement component C3b (enhances phagocytosis) • Antigen presentation within Class II MHC • Secrete IL-1 promoting T-cell differentiation and proliferation
Polymorphonuclear cells (neutrophils)	• Fc-receptor • C3b-receptor	• Bind Fc portion of immunoglobulin (enhances phagocytosis) • Bind complement component C3b (enhances phagocytosis)
NK cells	• Unknown	• Kills variety of target cells (e.g. tumor cells, virus-infected cells, transplanted cells)
Mast cells	• High affinity IgE Fc receptors	• Bind IgE and initiate allergic responses

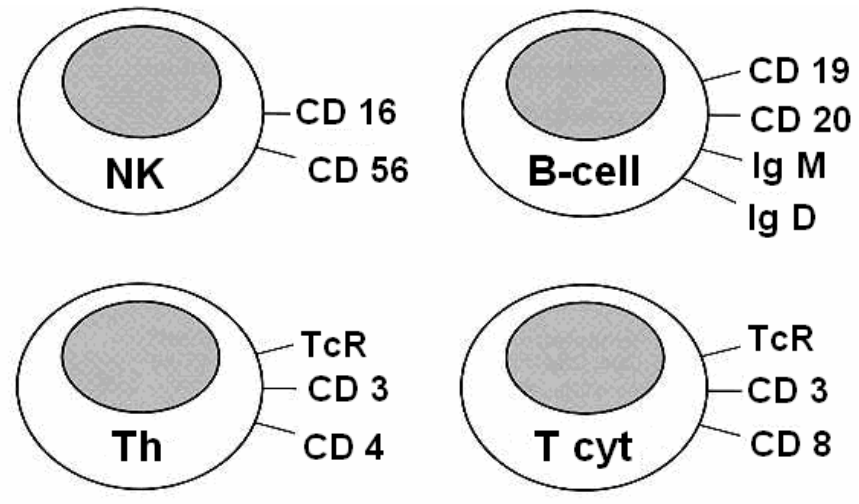


Figure 99 — Principal surface markers of lymphocyte population

T-cells

Originally, human T-cells were identified by their ability to bind to sheep red blood cells (SRBC); now known to be due to the presence of the **CD2** molecule on the T cell surface which acts as a receptor for certain molecules on the surface of the SRBC.

Later on, another group of membrane-anchored molecules was identified that comprises the polypeptide **CD3** complex. These molecules associate on the cell surface with the **T-cell antigen receptor** (TcR), a group of molecules used by T-cells to recognise and interact with an antigen in the form of a short string of amino acids. Since the TcR is not expressed on T-cells without the CD3 complex, the latter is now used to identify T-cells.

The **T-cell receptor molecule** is composed of two, disulfide-linked polypeptide chains; alpha and beta, each having separate constant and variable domains like immunoglobulins. The variable domain contains three hypervariable regions that are responsible for antigen recognition. Thus, just like the B-cell surface immunoglobulin provides antigen specificity to its B-cell, the TCR allows T-cells to recognize their particular antigenic determinant. However, T-cells cannot recognize antigen without help; the antigenic determinant must be presented by an appropriate MHC molecule. Upon recognition of a specific antigen, the signal is passed to the CD3 molecule and then into the T-cell, prompting T-cell activation and the release of lymphokines.

Furthermore, T-cells are subdivided according to the presence or absence of other surface molecules. Thus, in the blood, some T-cells express the **CD4** molecule while a distinct set express the **CD8** molecule. CD4+ T-cells were known as **helper cells** (e.g. they helped B-cells to produce antibody) and CD8+ T-cells as **cytotoxic cells** (they were capable of killing certain cells).

1. T-Helper cells: These cells are the primary regulators of T-cell and B-cell-mediated responses. They 1) Promotes *differentiation of B-cells* and *cytotoxic T-cells*; 2) express the **CD4 molecule**; 3) recognize foreign antigen complexed with **MHC class II molecules** on *B cells, macrophages or other antigen-presenting cells* (see figure 100).

2. T-Cytotoxic cells*:* These cells are cytotoxic against *tumor cells* and host *cells infected with intracellular pathogens*. These cells 1) usually express **CD8**, 2) destroy infected cells in an antigen-specific manner that is dependent on the expression of **MHC class I molecules**. Contact between the T-Cytotoxic cell and the target cell is required for lysis (the "kiss of death")

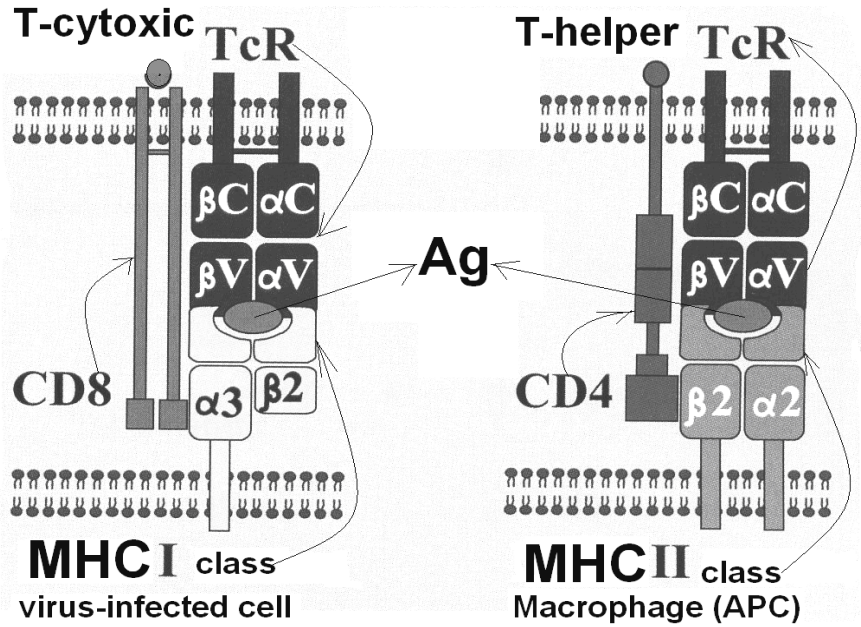


Figure 100 — Ag-presentation with MHC II and MHC I classes

3. T-Suppressor cells*:* These cells suppress the T and B-cell responses and express **CD8** molecules.

Naive cells which have not encountered antigen are referred to as T precursor or type 0 cells. Upon engagement of their antigen receptors, these cells secrete the **cytokine IL-2**. Other local cells may also be stimulated to release cytokines and these will influence the further development of the T-cells. The presence of **IL-18** and **IL-12** will lead to the development of *type 1 effector or memory cells*. The effector cells will secrete cytokines (most importantly **interferon gamma** – IFNg – and **tumour necrosis factor** – TNF) that stimulate a range of cells leading to cell-mediated immunity. Conversely, the presence of **IL-10** will lead to the development of *type 2 effector and memory cells*. The effector cells secrete **IL-4, IL-5, IL-6** and **IL-13** which influence the humoral immune response and affect the class of antibody produced in response to the antigen.

Natural killer cells*:* NK-cells are large granular lymphocytes that nonspecifically kill certain types of *tumor cells* and *virus-infected cells*. Killing by NK-cells is enhanced by cytokines such as **IL-2** and **IFN-gamma**. These lymphocytes do not express CD3, TcR or immunoglobulin, but display surface receptors (**CD16**) for the Fc-fragment of IgG antibodies. This type of CMI is called **antibody-dependent cell-mediated cytotoxicity** or **ADCC** (important defense against a variety of parasitic infections caused by *protozoa* and *helminthes*). This process is illustrated in figure 101.

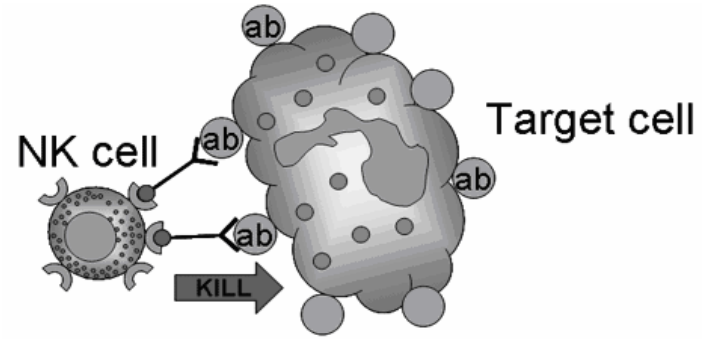


Figure 101 — Antibody-dependent cellular cytotoxicity

There are different classes of MHC genes and NK cells ‘‘expect’’ to see certain products of the class I genes on the target cell surface. If this molecule is not present (or is present at too low a concentration for recognition), the NK-cell will destroy the target cell through the action of molecules known as **perforins**.

Killer (K) cells have molecules on their surface that act as receptors (Fc-receptors) for a molecule known as immunoglobulin G (IgG) that can specifically recognise foreign particles in the host. Using these Fc-receptors, K-cells are able to bind to, and kill, cells that have immunoglobulin attached to them via their antigen-binding regions. Thus, if a cell is infected with a *virus* and some of the *viral proteins* are present in the cell membrane, antibodies formed against them will bind to the viral antigens on the surface of the cell. A killer cell can bind to this antibody, is thereby activated, and kills the virally infected (target) cell (Figure). This activity is known **antibody-dependent cellular cytotoxicity**.

B-cells

These cells differentiate into **plasma cells** to secrete antibodies. Pre-B cells derive from the common lymphoid progenitor. They are large and have constituents of immunoglobulins in their cytoplasm. They differentiate into immature and then mature B-cells, which have immunoglobulin and other molecules (e.g**. CD19, 20, 23, 24, 35** and **40**) inserted in their membranes.

B-cells comprise about 5–15% of circulating lymphocytes. A distinguishing feature of B-cells is the expression of immunoglobulin on their surface (**membrane Ig** or mIg), which acts *as the antigen receptor for the cell*. Like the TcR, the B-cell antigen receptor (BcR) consists of more than one group of molecules. In addition to mIg, the BcR comprises a duplex of molecules known as CD79a/b (formerly Iga and Igb) that have a single extracellular region and a cytoplasmic tail.

After exposure to an antigen (**antigenic stimulation or challenge**), B-cells proliferate and differentiate either into **plasma cells** or small, resting cells, which are able to respond next time the same antigen is encountered (**memory cells**).

Plasma cells secrete antibody molecules that have the same antigen specificity as the immunoglobulin found on the membrane of the parent cell. The genetic code for the antigen-binding region is highly variable, allowing each B-cell to produce molecules that recognise only a single, distinct antigen! The progeny of a B-cell all have the *same code for immunoglobulin* and so recognise the same antigen as the parent cell (i.e. have the **same antigenic specificity**).

The fact that the progeny of a single B-cell all produce antibody of the same specificity was exploited in the 1960s to produce a tool, which is widely used in many different scientific disciplines. B-cells, which had been stimulated by antigen, were mixed with tumour cells derived from B-cells that grow indefinitely but do not produce antibody.

By using a chemical to fuse the different cells, the resulting product was a B-cell that grew indefinitely and produced antibody of the same specificity as the original B cell. This meant an *endless supply* of highly specific, **monoclonal** (i.e. derived from a single cell and its daughters) **antibody** could be produced to virtually any antigen. *Such monoclonal antibodies have allowed us to identify molecules on the surface of cells* (CD molecules), to quantify both antibody and antigen, and are used in numerous immunological techniques.

Immune response

When tissue damage occurs as a result of trauma or infection, a complex series of cellular and biochemical events occur, which are designed to limit the spread of infection/degree of tissue damage, eliminate any microorganisms and repair the damaged tissue. Initially, these activities are limited to the innate immune response, i.e. they do not require specific identification of the precipitating organism but often a successful outcome requires the combined activity of both the innate and specific immune responses.

As a result of trauma and/or infection, cells in tissues are damaged and release their contents into the lymph, which bathes the tissues. One of the earliest consequences of tissue damage or infection is the **release of cytokines** from activated tissue macrophages and mast cells, in particular the **colonystimulating factors** (CSF). This causes the release of granulocytes and monocytes from the bone marrow providing a ready reserve of these front-line cells. Other cytokines released include those responsible for the increased expression of adhesion molecules on the endothelial cells lining the blood vessels and on leukocytes in the vicinity. The interaction of these adhesion molecules allows cells to attach to the endothelium. Other factors released at the site of damage/infection affect the vascular tone and the integrity of the endothelial layer, giving attached cells the opportunity to escape into the underlying tissues. This they do in response to certain cytokines known as **chemokines**.

Once at the site of infection/damage, the cells may actively phagocytose material recognised either through a group of molecules known as **pattern recognition receptors** or as a result of binding antibody or proteins of the **complement pathways** which encourage phagocytosis (i.e. they act as *opsonins*). As a result of phagocytosis, the organism may be destroyed by the action of antimicrobial enzymes found in the cellular granules. Alternatively, stimulation of the *oxidative (respiratory) burst* may lead to the production of reactive oxygen radicals, which can destroy the microorganism. This chemical and cellular response involves a number of other cells and molecules, which collectively are known as the **inflammatory response**.

Stimulation of a **specific immune response** requires the recognition of antigen by T-cells and B-cells. Usually, although B-cells can bind antigen directly, they require further signals provided by molecules on their surface binding to their respective signals. These signals may be provided by antigen-stimulated T-cells. Indeed, T-cell activation is also key to the cellular branch of specific immunity.

Cell-mediated immunity

Cell-mediated immunity includes all those specific immune responses in which antibody plays only a minor role.

T-cells in the periphery may be considered to be **naive, memory or effector cells**.

1. **Naive cells** are those that have not been stimulated by antigen since leaving the thymus.

2. By contrast, **memory cells** are those which have had antigen presented to them at least once and have returned to a resting state from which they can be activated on subsequent exposure to the same antigen. This group of cells is considered to be long-lived.

3. **Effector cells** are those T-cells that in response to presented antigen are able to carry out specialized functions such as the secretion of specific cytokines or the lysis of target cells. These cells derive from either naive or memory cells several days after antigenic stimulation. They are short-lived, in a highly activated state but require further stimulation before they can perform their effector function.

T-cells that generate CMI are present in the lymphoid tissues, blood and lymph. Due to constant recirculation between blood and lymph nodes via lymphatics and back to the blood, one T-cell circulates once in about 24 hours. Each carries receptors for the specific Ag with which it can react. **T-cell recognition of Ag only occurs when the Ag is associated with proteins of the MHC complex.** The T-cells have receptors (TcR) complementary to the complexed MHC determinant and the antigenic determinant.

When a T-cell recognizes presented antigen, the interaction between the **TcR–CD3–CD4** on the T-cell and the antigen–MHC complex on the APC results in a number of vital events, which ultimately lead to activation. As a result of interaction with the T-cell, the **antigen presenting cell (APC)** is stimulated to secrete **interleukin 1.** This results in the production of interleukin-2 and the expression of IL-2 receptors. Interleukin-2 is a polypeptide, which act on T-cells promoting their division.

Studies in mice have shown that naive cells only secrete interleukin-2 on initial stimulation while memory and effector cells may exhibit defined cytokine secretion profiles, e.g. T-Helper-1 **(TH1)** and T-Helper-2 **(TH2) cytokine profiles**. TH1 cells secrete lymphokines that activate macrophages and mediate **delayed type hypersensitivity responses**. TH2 cells secrete lymphokines that stimulate B cell development and may help activate T cytotoxic cells to their full cytotoxic capacity. TH1 cells and TH2 cells recognize Ag in association with MHC II (as displayed by macrophages and other APCs); TC-cells recognize Ag on cells complexed with MHC I (as displayed by altered self cells).

Subsets of T-cells have different patterns of cytokine production which stimulate different types of immune response (figure 102).

TH1 cells "see" foreign Ag on the surface of APC in the context of MHC II. Mainly, TH1 cells produce IL-2, gamma IFN and lymphotoxin. This results in macrophage activation and the delayed-type hypersensitivity (DTH) reaction, and in help for TC cell activation.

TH2 cells also see foreign Ag on the surface of APC in the context of MHC II. Their response is to secrete IL-4, IL-5, IL-6, IL-10 and IL-13 that help activate B cells, provide help for the production of IgE that attaches to mast cells, and promote mast cell and eosinophil activation.

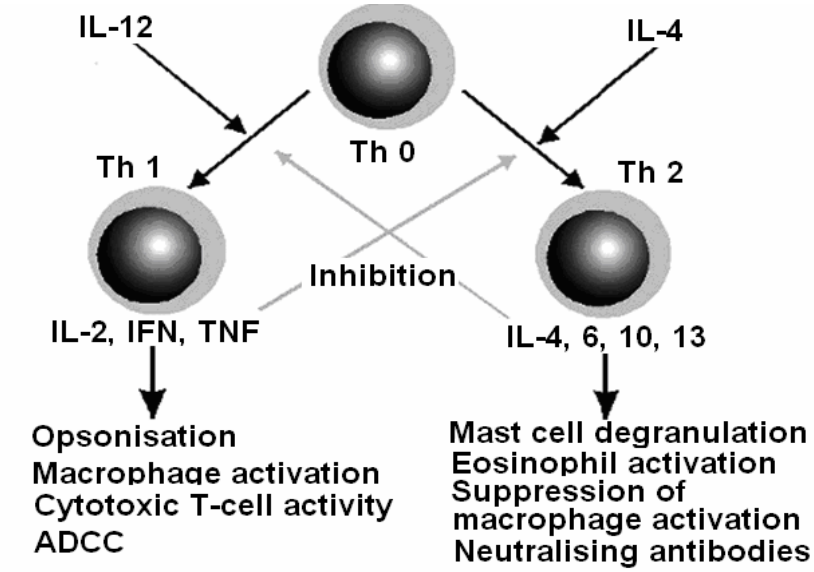


Figure 102 — Cytokine secretion patterns of T helper subsets

Both types of TH cells develop under most conditions but their ratios and the predominance of certain lymphokines can vary, and this may mediate the pathology and outcome of certain bacterial infections.

Involvement of macrophages in mediation of CMI

During induction of the cell-mediated immune response, **macrophages** play their usual role in the presentation of Ag to T-helper cells and in producing cytokines that are involved in the initiation of immune reactions (see figure 103). In addition, macrophages play a role in the expression of CMI. Many of the lymphokines produced by TH cells are aimed at attraction, entrapment and activation of macrophages at the site of the reaction.

One of these lymphokines, **Gamma Interferon**, causes the local macrophage population to develop an increased number of lysosomes and also increased secretion of microbicidal products. Oxygen-dependent killing mechanisms of the macrophage are stimulated, and the macrophage develops increased power to ingest and kill microorganisms. Such lymphokine-stimulated macrophages are referred to as *"angry" or activated macrophages.*

Function of cytotoxic T-lymphocytes

Macrophage involvement in CMI may be part of the pathology of certain diseases. Where there is difficulty in elimination an intracellular parasite (e.g. the tuberculosis bacillus) the chronic CMI response to local antigens leads to the accumulations of densely-packed macrophages which release fibrinogenic factors and stimulate the formation of granulation and fibrosis. The resulting structure, called a **granuloma**, actually represents an attempt by the host to isolate a persistent infection.

TC (cytotoxic) cells can destroy cells bearing new antigens on their surfaces (as might result in a viral infection, a tumor cell, or an infection by a bacterial intracellular parasite). TC cells exert their cytotoxic activity when they are in *physical contact* with cells bearing new Ag and MHC I protein. Contact between the TC cell and the target cell is required for lysis, although the exact mechanism of lysis is not known.

TC cells generally respond to Ag in association with MHC I proteins on the surface of a target cell. If they responded to Ag by itself, they could react with it when it was free in extracellular fluids, and their cytotoxic activity would be triggered off with no purpose. As stated above, almost all host cells, including macrophages, display MHC I. Hence, an effector TC cell can destroy a macrophage which is otherwise carrying out a useful function by presenting Ag to TH lymphocytes as part of the AMI or CMI responses. Usually, the time course of the response is such that TH cells have already developed and have carried out their (helping) function when TC cells begin to become active.

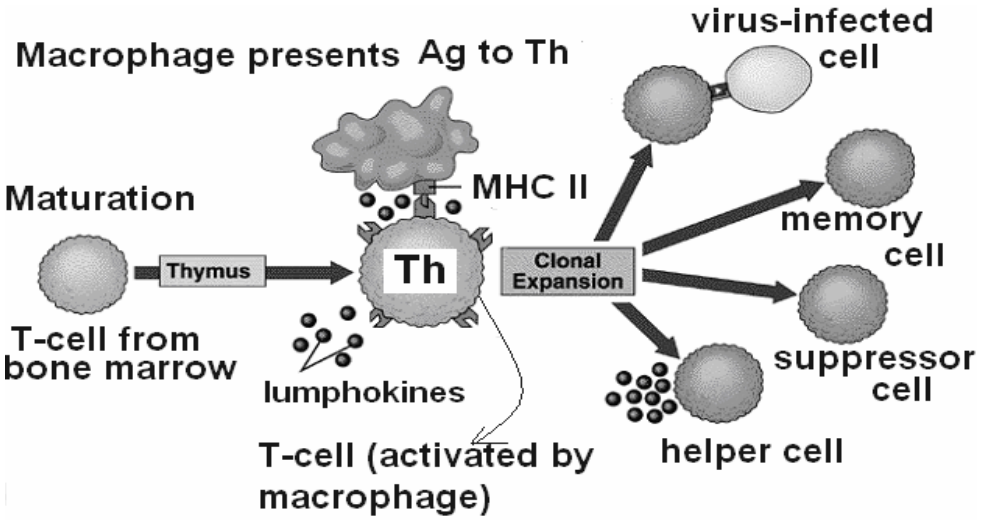


Figure 103 — T-cell activation (CMI)

Delayed type hypersensitivity

Delayed-type hypersensitivity reactions usually present themselves as allergic reactions. Such allergic reactions generally require about 24 hours to develop following a secondary exposure to Ag. This time is required for the circulating TH cells (actually memory cells) to encounter the Ag and to begin producing lymphokines, and to attract macrophages and TC cells to the site, for these cells are the real mediators of the allergic reaction. The phagocytic and cytolytic activities of these cells are responsible for the localized tissue destruction which occurs.

One of the best known examples of the delayed-type hypersensitivity reaction is the **Mantoux (tuberculin) test** which is utilized to determine current or previous infection by the tubercle bacillus (*Mycobacterium tuberculosis*). A small amount of Ag called the purified protein derivative (PPD), derived from the cell wall of the bacterium, is injected subcutaneously usually just under the skin of the forearm. The test is evaluated after 24-48 hours. A **positive test** is an allergic response (an "urticarial weal") at the site of the injection, which might look like a swollen reddened area about the size of a quarter. A **negative test** is no reaction. A positive test does not mean that the individual has an active case of tuberculosis, but that the individual has at least been exposed to the tubercle bacillus or one of its products sufficiently to have undergone a primary immune response. Hence, an individual exhibiting a positive test may have active tuberculosis, may have an unapparent (subclinical) form of the disease, may have previously had the disease, or possibly may have been artificially immunized against the disease.

Other aspects of cell-mediated immunity

Another class of cytotoxic lymphocytes distinct from TC cells may be stimulated during the cell-mediated immune response. These are referred to as **Natural Killer** or **NK cells**. NK cells are found in blood and lymphoid tissues, especially the spleen. They do not bear T cell (or B cell) markers. Like TC cells, they are able to recognize and kill cells that are displaying a new Ag on their surfaces, but unlike TC cells, they do not display TCR and they are not MHC-restricted.

The existence of NK specificity is demonstrated by the phenomenon of "cold target inhibition"; one NK target cell type can inhibit lysis of a different NK target type by competing for effector cells, whereas cells that are not NK targets do not compete. NK cells are present in an animal in the absence of antigenic stimulation, and it is for this reason that they are referred to as "natural" killers. They might also be considered part of the constitutive defenses; however, NK cells become activated in a CMI response by T cell lymphokines, including Interleukin-2 and Gamma Interferon.

Some NK cells are thought to be an immature form of a T-lymphocyte, but various other types of cells including macrophages, neutrophils and eosinophils, display NK activity. Some NK cells have surface receptors (**CD16**) for the Fc portion of IgG. They bind to target cells by receptors for the Fc portion of antibody that has reacted with antigen on the target cell. This type of CMI is called **antibody-dependent cell-mediated cytotoxicity** or ADCC. ADCC is thought to be an important defense against a variety of parasitic infections caused by protozoa and helminths.

Antibody-mediated immunity

The B-cell response to an antigenic challenge results in the production of specific antibody. The type of antibody and the kinetics of its production depend on whether or

not it is the first time that the host has seen the antigen (Figure 104). In a secondary response, not only is the response much more rapid and of much greater magnitude but also the antibodies show much higher affinity to the eliciting antigen. This change in antibody class and affinity is due to B-cell maturation, differentiation and activation.

Induction of primary immune responses: Antibody level has to build up to effective physiological concentration to render host resistant. It may take several days or weeks to reach a level of effective immunity, even though this immunity may persist for many months, or years, or even a lifetime, due to the presence of the antibodies. In natural infections, the inoculum is small, and even though the antigenic stimulus increases during microbial replication, only small amounts of antibody are formed within the first few days, and circulating antibody is not detectable until about a week after infection.

Induction of a secondary immune response (**secondary exposure to antigen**): Larger amounts of antibodies are formed in only 1-2 days. This is due to the activities of **specific memory B-cells** or **memory T-cells** which were formed during the primary immune response. These memory cells, when stimulated by homologous Ag, "remember" having previously seen the Ag, and are able to rapidly divide and differentiate into effector cells. Stimulating memory cells to rapidly produce very high levels of persistent circulating antibodies is the basis for giving "booster"-type vaccinations to humans and pets.

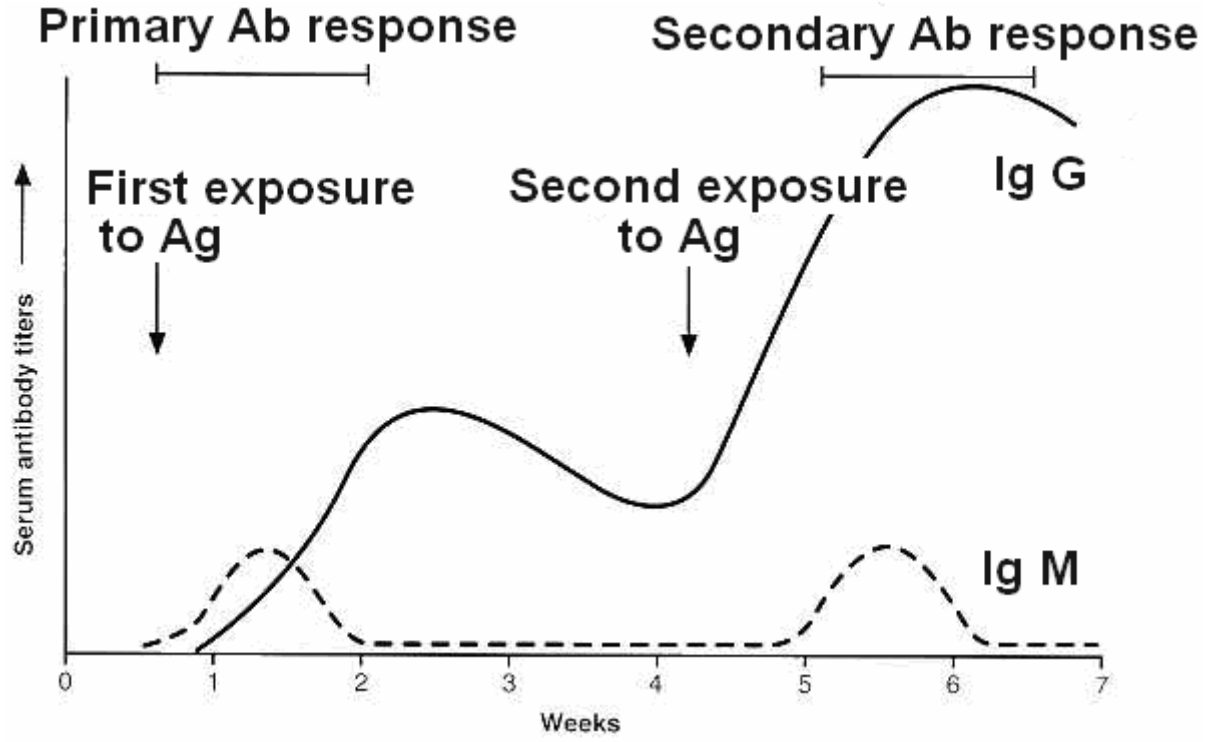


Figure 104 — Primary and secondary immune response

The antibody formed in the primary response is **predominantly IgM** and in the **secondary response IgG**. The early antibody is more specific but less avid than the late antibody.

First exposure to an antigen the immune response develops gradually over a period of days, reaches a low plateau within 2-3 weeks, and usually begins to decline in a relatively short period of time. When the antigen is encountered a second time, a secondary (memory) response causes a rapid rise in the concentration of antibody, reaching a much higher level in the serum, which may persist for a relatively long period of time. This is not to say that a protective level of antibody may not be reached

by primary exposure alone, but usually to ensure a high level of protective antibody that persists over a long period of time, it is necessary to have repeated antigenic stimulation of the immune system.

Activation of B-cells and production of antibodies

Immune response to an antigen is brought about by three types of cells — *antigen processing cells* (APC — principally macrophages and dendritic cells), T-cells and B-cells. The **first step** is the capture and processing of the antigens by APC and their presentation, in association with the appropriate MHC molecule, to T-cells. While this step is essential for most antigens (*T-cell dependent antigens* such as proteins and erythrocytes), in the case of *T-cell independent antigens*, such as polysaccharides and other structurally simple molecules with repeating epitopes, antibody production does not require T-cell participation.

Only when the processed antigen is presented on the surface of APC, in association with MHC molecules, to the T-cell carrying the receptor (TcR) for the epitope is the T-cell able to recognise it. In the case of T-helper (CD4), the antigen has to be presented complexed with MHC Class II and for T-cytotoxic (CD8) with MHC Class I molecules. B-cells, which possess surface Ig and MHC Class II molecules, can also present antigens to T-cells, particularly during the secondary response.

The T-helper cell requires **two signals for activation**. The first signal is a combination of the *T cell receptor* (TcR) with the MHC Class II-complexed antigen. The second signal is *interleukin-I* (IL-1) which is produced by the APC. The activated T_H cell forms inter-leukin-2 and other cytokines required for B cell stimulation. These include IL-4, IL-5 and IL-6 which act as B cell growth factor (BCGF) and the B cell differentiation factor (BCDF) that activate B-cells which have combined with their respective antigens to clonally proliferate and differentiate into antibody-secreting plasma cells. A small proportion of activated B-cells become long lived memory cells producing a secondary type of response to subsequent contact with the antigen (see figure 105).

As the immune response to a T-dependent antigen matures the predominant class of antibody changes from **IgM** to **IgG, IgA or IgE**. This may be explained by the **genetic switch hypothesis**. This ‘‘class switching’’ allows a cell to produce a different class of immunoglobulin (with different biological properties) while retaining the same specificity for antigen. **Interleukin 4** has been shown to cause the switch to **IgG** and **IgE** in humans.

However, the switching process requires the presence of monocytes and physical interactions with T-cells since isolated B-cells and purified IL-4 do not show class switching. The physical contact with T-cells is thought to require the expression of a surface antigen on activated cells, which is the ligand for the CD40 molecule expressed on B cells (CD40L or CD154). Indeed, antibodies to CD40 in association with IL-4 have been shown to negate the requirement for T cells in IgE switching.

Thus: 1) the **B-cell response** to an antigen, i.e. the type of antibody produced is dependent upon whether or not it is the first time that the antigen has been seen. 2) Secondary responses are much greater and much more rapid than primary responses. In secondary responses, the antibody shows much higher affinity for the antigen. 3) B-cells bind antigen **directly**, but require interaction with T-cells in the form of **CD40–CD154 co-ligation** to provide the intracellular signals for differentiation and proliferation. 4) **T-independent antigens** may stimulate B-cells without the need for T-cell interaction but the cytokines produced by activated T-cells are still necessary. 5) **Mem-**

ory does not develop to T-independent antigens and so the antibody response is characteristically **IgM in nature**. 6) Class switching (**from IgM production to either IgG, A or E**) requires *T-cell contact* and *specific cytokines*.

Monoclonal antibodies

A single antibody forming cell or **clone** produces antibodies specifically directed against a single antigen or antigenic determinant only. However, antibodies produced ordinarily by infection or immunisation are polyclonal because natural antigens have multiple epitopes or antigenic determinants, each of which generates separate clones of lymphocytes. This results in antisera containing immunoglobulins of different classes with specificities against different epitopes of the antigen. On the other hand, when a clone of lymphocytes or plasma cells undergoes selective proliferation, as in multiple myeloma, antibodies with a single antigenic specificity accumulate. Such antibodies produced by a single clone and directed against a single antigenic determinant are called monoclonal antibodies. *Monoclonal antibodies* are very useful tools for diagnostic and research techniques.

Fate of antigen in tissues

The manner in which an antigen is dealt with in the body depends on factors such as the *physical* and *chemical nature* of the antigen, its *dose* and *route of entry* and whether the antigenic stimulus is primary or secondary.

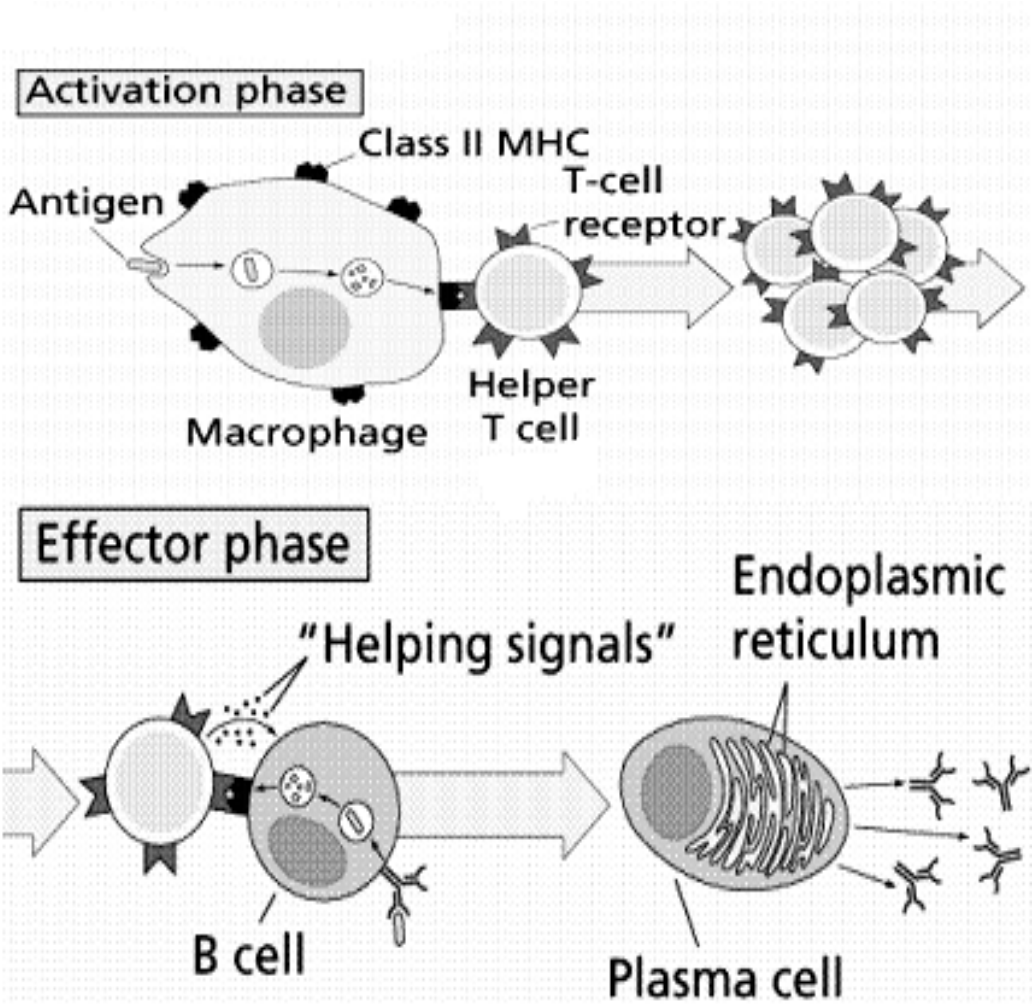


Figure 105 — Antibody-mediated response

Antigens introduced intravenously are rapidly localised in the spleen, liver, bone marrow, kidneys and lungs. They are broken down by the reticuloendothelial cells and excreted in the urine, about 70–80 per cent being thus eliminated within one or two days.

In contrast, **antigens introduced subcutaneously** are mainly localised in the draining lymph nodes, only small amounts being found in the spleen. Particulate antigens are removed from circulation in two phases. The first is the **nonimmune phase** during which the antigen is engulfed by the phagocytic cells, broken down and eliminated. With the appearance of the specific antibody, the **phase of immune elimination** begins, during which antigen-antibody complexes are formed and are rapidly phagocytosed, resulting in an accelerated disappearance of the antigen from circulation.

With *soluble antigens*, three phases can be recognised — **equilibration, metabolism** and **immune elimination**. The phase of equilibration consists of diffusion of the antigen to the extravascular spaces. During the metabolic phase, the level of the antigen falls due to catabolic decay. During the phase of immune elimination, there is rapid elimination of the antigen with the formation of antigen-antibody complexes. Such complexes can cause tissue damage and may be responsible for 'immune complex diseases' such as serum sickness.

The speed of elimination of an antigen is related to the speed at which it is metabolised. Protein antigens are generally eliminated within days or weeks, whereas polysaccharides which are metabolised slowly, persist for months or years. Pneumococcal polysaccharide, for instance, may persist up to 20 years in human beings, following a single injection.

Bacterial mechanisms to overcome host immune defenses

● **Immunological tolerance to a bacterial Ag.** Tolerance is a property of the host in which there is an immunologically-specific reduction in the immune response to a given Ag. Tolerance to a bacterial Ag does not involve a general failure in the immune response but a particular deficiency in relation to the specific antigen(s) of a given bacterium. If there is a depressed immune response to relevant antigens of a parasite, the process of infection is facilitated. Tolerance can involve either AMI or CMI or both arms of the immunological response. Tolerance to an Ag can arise in a number of ways, but three are possibly relevant to bacterial infections.

1. **Fetal exposure to Ag**. If a fetus is infected at certain stages of immunological development, the microbial Ag may be seen as "self", thus inducing tolerance to the Ag which may persist even after birth.

2. **High persistent doses of circulating Ag**. Tolerance to a bacterium or one of its products might arise when large amounts of bacterial antigens are circulating in the blood.

3. **Molecular mimicry**. If a bacterial Ag is very similar to normal host "antigens", the immune responses to this Ag may be weak giving a degree of tolerance. Resemblance between bacterial Ag and host Ag is referred to as molecular mimicry. In this case the antigenic determinants of the bacterium are so closely related chemically to host "self" components that the immunological cells cannot distinguish between the two and an immune response cannot be raised. Some bacterial capsules are composed of polysaccharides (hyaluronic acid, sialic acid) so similar to host tissue polysaccharides that they are not immunogenic.

● Bacteria may be able to coat Ag with host proteins such as **fibrin**, **fibronectin**, or even **Ig molecules**. In this way they are able to hide their own antigenic surface components from the immunological system.

S. aureus produces cell-bound **coagulase** and **clumping factor** that cause fibrin to clot and to deposit on the cell surface. It is possible that this disguises the bacteria immunologically so that they are not readily identified as antigens and targets for an immune response.

Protein A produced by *S. aureus*, and the analogous **Protein G** produced by *Streptococcus pyogenes*, bind the Fc portion of immunoglobulins, thus coating the bacteria with antibodies and canceling their opsonizing ability.

● Some pathogens (mainly viruses and protozoa, rarely bacteria) cause **immunosuppression** in the infected host. This means that the host shows depressed immune responses to antigens in general, including those of the infecting pathogen (chronic bacterial infections such as leprosy and tuberculosis).

● Macrophages support the growth of the bacteria and at the same time gives them protection from immune responses (**intracellular pathogens**). *Streptococcus mutans* can initiate **dental caries** at any time after the eruption of the teeth, regardless of the immune status of the host. Either the host does not undergo an effective immune response or secretory IgA plays little role in preventing colonization and subsequent plaque development. *Vibrio cholerae* multiplies in the GI tract where the bacteria elaborate a toxin which causes loss of fluids and diarrhea in the host which is characteristic of the disease **cholera**. IgA antibodies against cellular antigens of the cholera vibrios are not completely effective in preventing infection by these bacteria as demonstrated by the relative ineffectiveness of the cholera vaccine prepared from phenol-killed vibrios. The carrier state of **typhoid fever** results from a persistent infection by the typhoid bacillus, *Salmonella Typhi*. The organism is not eliminated during the initial infection and persists in the host for months, years or a life time. In the carrier state *S. Typhi* is able to colonize the biliary tract away from the immune forces, and be shed into urine and feces.

● Induction of **ineffective antibody.** In the case of *Neisseria gonorrhoeae* the presence of antibody to an outer membrane protein interferes with the serum bactericidal reaction and in some way compromises the surface defenses of the female urogenital tract. Increased susceptibility to reinfection is highly correlated with the presence of circulating antibodies.

● Some pathogens produce **enzymes** that destroy antibodies (protein A produced S. aureus agglutinates IgG). *Neisseria gonorrhoeae*, *N. meningitidis*, *Streptococcus pneumoniae* and *Streptococcus mutans*, which can grow on the surfaces of the body, produce **IgA proteases** that inactivate secretory IgA by cleaving the molecule at the hinge region, detaching the Fc region of the immunoglobulin. Soluble forms of **Protein A** produced *S. aureus* agglutinate immunoglobulin molecules and partially inactivate IgG.

● Many pathogenic bacteria exist in nature as multiple antigenic types or serotypes, meaning that they are variant strains of the same pathogenic species. For example, there are multiple serotypes of *Salmonella Typhimurium* based on differences in cell wall (O) antigens or flagellar (H) antigens. There are 80 different antigenic types of *Streptococcus pyogenes* based on M-proteins on the cell surface. There are over one hundred strains of *Streptococcus pneumoniae* depending on their capsular polysaccharide antigens. Based on minor differences in surface structure chemistry there are multiple serotypes of *Vibrio cholerae*, *Staphylococcus aureus*, *Escherichia coli*, *Neisseria gonorrhoeae* and an assortment of other bacterial pathogens. Antigenic variation is prevalent among pathogenic viruses as well.

● Antibodies that are bound to bacterial surfaces will activate **complement** by the *classical pathway* and bacterial polysaccharides activate complement by the *alternative pathway*. Bacteria in serum and other tissues, especially Gram-negative bacteria, need protection from the antimicrobial effects of complement.

One role of **capsules** in bacterial virulence is to protect the bacteria from complement activation and the ensuing inflammatory response. Polysaccharide capsules can hide bacterial components such as LPS or peptidoglycan which can induce the alternate complement pathway. Some bacterial capsules are able to inhibit formation of the C3b complex on their surfaces, thus avoiding C3b opsonization and subsequent formation of C5b and the membrane attack complex (MAC) on the bacterial cell surface.

Hypersensitivity

Immunity was originally considered a protective process, helping the body to overcome infectious agents and their toxins. This however is only one aspect of the broad phenomenon of immunity which includes all manner of specific responses to antigens. Immune response may sometimes be injurious to the host. Sensitised individuals respond to subsequent antigenic stimuli in an inappropriate manner, leading to tissue damage, disease or even death.

The term **hypersensitivity** refers to the *injurious consequences in the sensitised host, following contact with specific antigens*. In the protective processes of immunity, the focus of attention is the antigen and what happens to it — for example, killing of a bacterium or neutralization of a toxin. In hypersensitivity, on the other hand, antigens are of little concern and often, they are innocuous or bland substances such as serum proteins or pollen. *Hypersensitivity is concerned with what happens to the host as a result of the immune reaction*. Considerable confusion is attached to the use of the term “allergy”. As originally used by von Pirquet, **allergy** meant an altered state of reactivity to an antigen, and included both types of immune responses, protective as well as injurious. It is still used in this broad sense by some. Others use the term “allergy” to mean all immune processes harmful to the host, such as hypersensitivity and autoimmunity. Allergy is probably most commonly used as a synonym for hypersensitivity. It is sometimes employed in a narrow sense to refer to only one type of hypersensitivity, namely 'atopy'. For induction of hypersensitivity reactions, the host should have had contact with the antigen (allergen). The initial contact sensitises the immune system, leading to the priming of the appropriate B or T-lymphocytes. This is known as the “**sensitising**” or “**priming**” dose. Subsequent contact with the allergen causes manifestations of hypersensitivity. This is known as the “**shocking**” dose.

There are four different types of hypersensitivities that result from different alterations of the immune system. These types are classified as:

Type I: **Immediate hypersensitivity**
Type II: **Cytotoxic hypersensitivity**
Type III: **Immune complex hypersensitivity**
Type IV: **Delayed hypersensitivity**

Type I hypersensitivity

Type I hypersensitivity reactions are becoming more common and you would recognise them as the signs and symptoms associated with allergic responses/

Whilst the allergens (antigens) that stimulate these reactions are diverse, the outcome of exposure to any allergen is similar. What determines whether or not an individual will respond normally to an antigen is not fully understood. Clearly there is a genetic influence, since family studies have shown that if both parents are allergic

an offspring has a 75% chance of being allergic. Type I or **Immediate Hypersensitivity** can be illustrated by considering the following experiment:

First, a guinea pig is injected intravenously with an antigen. For this example, bovine serum albumin (BSA, a protein) will be used. After two weeks, the same antigen will be reinjected into the same animal. Within a few minutes, the animal begins to suffocate and dies by a process called *anaphylactic shock*.

Instead of reinjecting the immunized guinea pig, serum is transferred from this pig to a "naive" (unimmunized) pig. When this second guinea pig is now injected with BSA, it also dies of anaphylactic shock. However, if the second pig is injected with a different antigen (e.g. egg white albumin), the pig shows no reaction.

If immune cells (T-cells and macrophages instead of serum) are transfered from the immunized pig to a second pig, the result is very different; injection of the second pig with BSA has no effect.

These results tell that:

1. The reaction elicited by antigen occurs very rapidly (hence the name "immediate hypersensitivity").
2. The hypersensitivity is mediated via serum-derived components (i.e. antibody).
3. The hypersensitivity is antigen-specific (as one might expect for an antibody-mediated reaction).

During the **first exposure** to allergen, contact is made with B-cells that differentiate and produce **IgE antibody**. The IgE against the allergen is released into the bloodstream and eventually binds to Fc receptors on **mast cells** and **basophils**. The mast cells are now sensitized, laying in wait for the second exposure (see figure 106).

Upon **contact with allergen a second time**, it now attaches to IgE present on mast cells causing **degranulation**. On the cellular level, the granules present in the cytoplasm migrate to the cellular membrane and spill out their contents into the surrounding area. This results in the release of histamine, slow reacting substance of anaphylaxis (SRS-A), heparin, prostaglandins, platelet-activation factor (PAF), eosinophil chemotactic factor of anaphylaxis and proteolytic enzymes. This cocktail of proteins are the **mediators of inflammation** and they trigger a number of **physiological responses** including smooth muscle contraction, an increase in vascular permeability and mucous secretion.

In its most severe form, **systemic anaphylaxis**, there is a *generalized response*. Exposure to allergen causes an immediate, large amount of mast cell response over a short period of time. This results in large concentrations of the mediators of inflammation being released all at once. Individuals experiencing systemic anaphylaxis will have trouble breathing due to smooth muscle contraction causing the closing of the bronchioles in the lungs. Arterioles will also dilate, resulting in a drop in blood pressure and capillary permeability that causes a loss of fluid into tissues. Victims of this response can die within minutes from reduced blood return through the veins, asphyxiation, low blood pressure and circulatory collapse leading to shock. Quick treatment with **epinephrine** (increases blood flow and inactivates mast cells) and **antihistamines** (combat histamines) is essential to prevent death.

Common allergens in this type of reaction are penicillin, passively administered antisera and insect venom from bees or wasps.

Localized anaphylaxis (**atopic allergy**) is a less severe form of anaphylaxis, whose symptoms depend primarily upon how the allergen enters the body. In **hay fever** (allergic rhinitis) the allergen enters the upper respiratory tract.

Common allergens in hay fever include pet dander, pollen, fungal spores and household dust mites. Exposure to these particles causes the typical symptoms of hay fever, i.e., runny nose, itchy eyes, coughing and sneezing, most of which are indicative of the action of mast cells. Treatment typically involves the use of **antihistamines** to block the action of histamine released by mast cells.

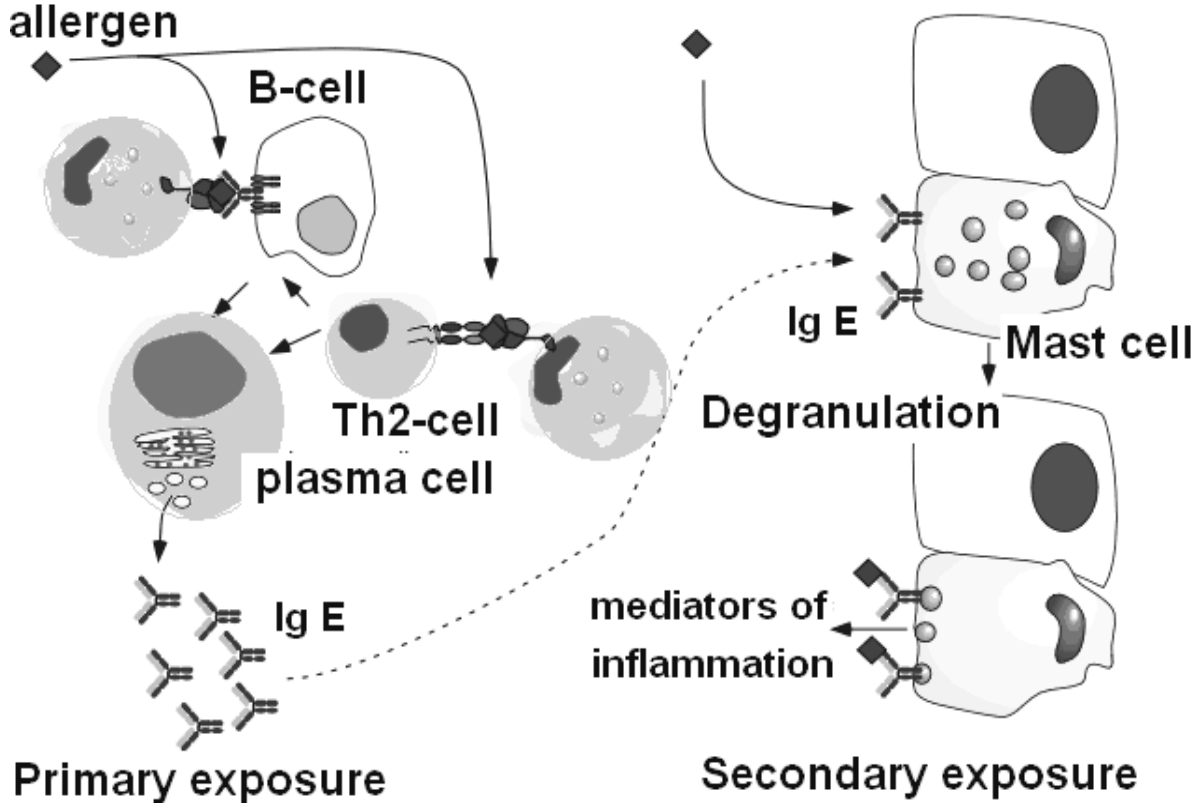


Figure 106 — Anaphylactic reaction

Bronchial asthma results when the site of immunological response is the lower respiratory tract. The same allergens that irritate the upper respiratory tract in this case cause the symptoms of asthma. Mucus accumulates in the alveoli (of the lungs and smooth muscle contraction of the bronchi narrows the airways and causes the characteristic wheezing of asthma. Bronchodilators that relax the bronchial muscles and expectorants that clear mucous plugs in the lungs can relieve most of the symptoms of asthma, but it is still a serious illness that can be fatal if treatment is delayed for too long.

A third type of localized anaphylaxis can be caused by allergens that enter through the digestive tract. Eruptions of the skin called hives are a strong indication of a **true food allergy**. Once a food allergy is established, it is usually permanent and the only option is to diminish the reaction with antihistamines or to avoid the suspect food altogether. Wheat, peanuts, soybeans, cows milk, eggs and less often shellfish are common causes of food allergies.

Skin testing can be done to identify the cause of common allergies as shown in figure 107. These tests consist of inoculation with small amounts of potential allergens and after a suitable incubation period (usually 24 hours), observation for the appearance of a hard, red welt indicating a positive response. Once the responsible allergen is known, the individual has three choices. The first and most obvious is to avoid the allergen and for most food allergies, this is the preferred choice.

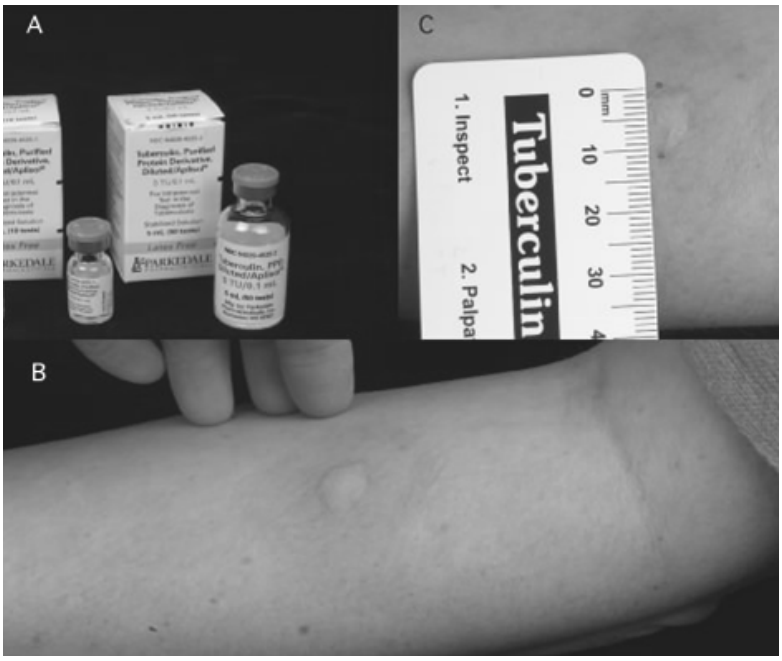


Figure 107 — Skin testing (Tuberculin allergen is available commercially (A). A small amount of allergen is injected under the skin. If an individual is allergic to a substance immune response will manifest in the form of redness and swelling (B). The size of the reaction is measured and this is used to gauge a persons exposure to tuberculosis (C). This individual has been exposed to TB in the past).

For allergens that are difficult or impossible to avoid (e.g., pollen, mold, dust mites etc.), treatment with antihistamines is useful. In severe cases, where the first two choices are unsatisfactory, it is sometimes possible to desensitize an individual by repeated injections with the offending allergen. These subcutaneous injections actually cause the elicitation of a second immune response that raises IgG antibodies to the allergen. The desensitization works because the IgG is able to react with the allergen and remove it before the IgE on the mast cells can react with it. Suppressor T-cells may also play a role in desensitization.

Type II hypersensitivity

Type II or **Cytotoxic Hypersensitivity** also involves antibody-mediated reactions. However, the immunoglobulin class (isotype) is generally **IgG**. In addition, this process involves **K-cells** rather than mast cells. K-cells are, of course, involved in **antibody-dependent cell-mediated cytotoxicity** (ADCC). Type II hypersensitivity may also involve **complement** that binds to cell-bound antibody. The difference here is that the antibodies are specific for (or able to cross-react with) "self" antigens. When these circulating antibodies react with a host cell surface, tissue damage may result.

There are many examples of Type II hypersensitivity. These include: **Pemphigus:** IgG antibodies that react with the intracellular substance found between epidermal cells. **Autoimmune hemolytic anemia (AHA):** This disease is generally inspired by a drug such as penicillin that becomes attached to the surface of red blood cells (RBC) and acts as hapten for the production of antibody which then binds the RBC surface leading to lysis of RBCs. **Goodpasture's syndrome:** Generally manifested as a glomerulonephritis, IgG antibodies that react against glomerular basement membrane surfaces can lead to kidney destruction.

Type III hypersensitivity

Type III or **Immune Complex hypersensitivity** involves circulating antibody that reacts with free antigen. These circulating complexes can then become deposited on tissues. **Tissue deposition** may lead to reaction with complement, causing tissue damage. Figure 108 depicts an immune complex hypersensitivity.

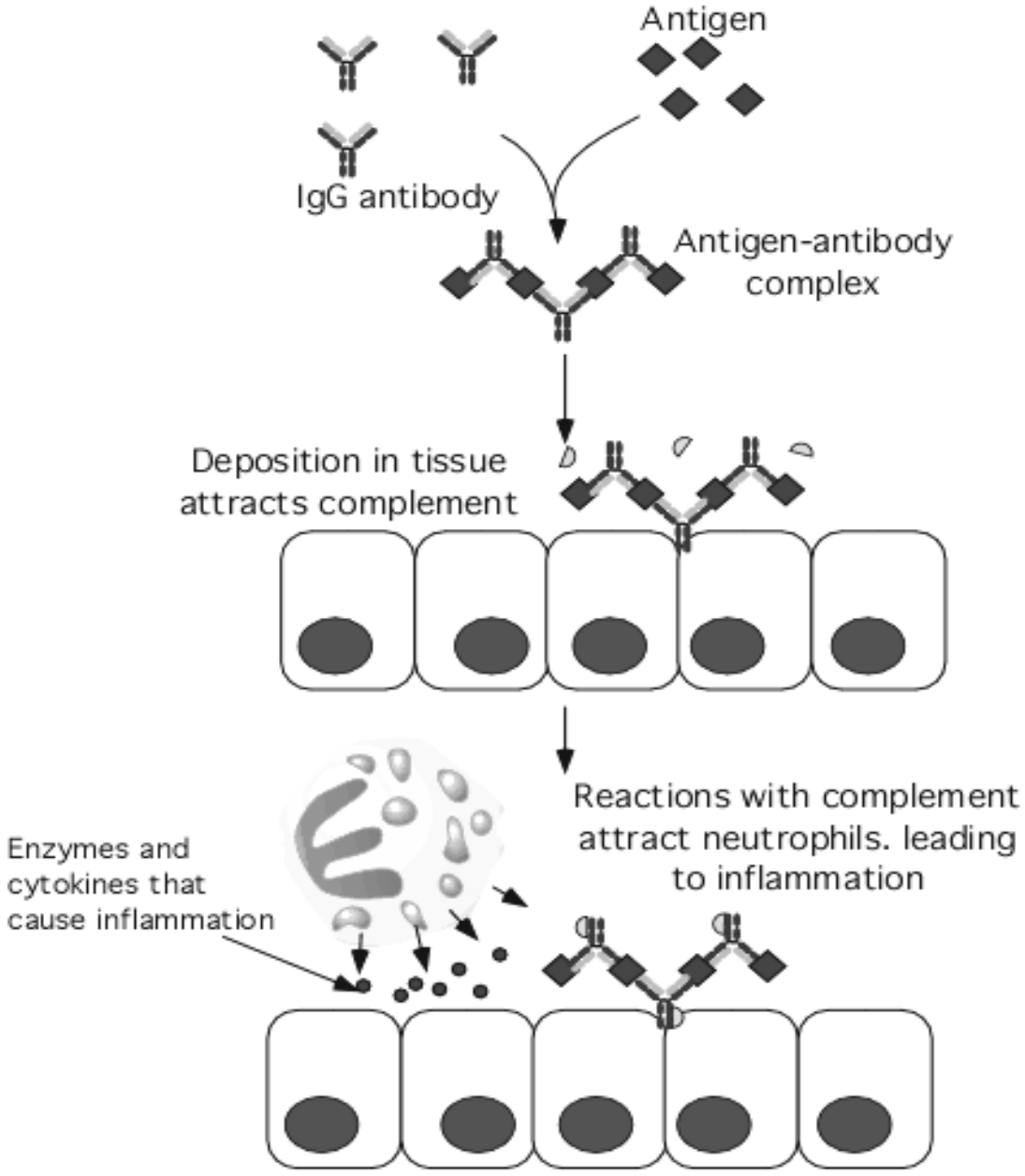


Figure 108 — Immune complex-mediated hypersensitivity

This type of hypersensitivity develops as a result of *systematic exposure* to an antigen and is dependent on: a) type of antigen and antibody and b) size of the resulting complex. More specifically, complexes that are too small remain in circulation; complexes too large are removed by the glomerulus; intermediate complexes may become accumulate in the different organs. Accumulation of type III hypersensitivity complexes is most commonly observed in the kidneys, blood vessels, the joints and the skin.

Three types of diseases can lead to immune complex hypersensitivity. First, **chronic infection with a virus, bacteria or protozoa**, along with a weak antibody response, will eventually lead to the deposition of immune complexes (conglomerations of antibody and antigens) and trigger this allergic response. A second type of this condition stems from antibody raised against self-antigens. Since the antigens are not removed in this case, immune complexes are constantly being formed and this is one of the major damaging side effects of autoimmune disease such as systemic **lupus erythematosus** and **rheumatoid arthritis**.

Third, constant chronic exposure to a particular antigen at a body surface can cause type III hypersensitivity reactions. **Farmer's lung** is an example of this type of disease. Repeated exposure to fungal spores present in moldy hay elicits an immune response and results in the formation of IgG antibody directed against the spores. Subsequent exposure causes the formation of IgG-spore immune complexes that accumulate in the alveoli of the lungs and cause inflammation leading to lung tissue damage. Other examples of type III hypersensitivity and the allergen that precipitates the problem include, **cheese washer's disease** (*Penicillium casei*), Furrier's lung (fox fur protein) and Maple bark stripper's disease (*Cryptostroma* spores).

One example of Type III hypersensitivity is **serum sickness**, a condition that may develop when a patient is injected with a large amount of e.g. antitoxin that was produced in an animal. After about 10 days, anti-antitoxin antibodies react with the antitoxin forming immune complexes that deposit in tissues.

Type III hypersensitivities can be ascertained by intradermal injection of the antigen, followed by the observance of an **"Arthus" reaction** (swelling and redness at site of injection) after a few hours.

Type IV hypersensitivity

Cell-mediated or delayed hypersensitivity is related to the T-cell response to an allergen. The major factor causing the delay is the time required for T-cells that respond to the allergen to migrate to and accumulate at the site of exposure. Delayed hypersensitivity reactions typically begin to appear 24 hours after exposure and reach their maximum intensity in 1 to 3 days. Antigens from microorganisms as well as certain chemicals can cause these types of reactions. Figure 109 summarizes the steps in a cell mediated allergic reaction.SYS

In delayed type hypersensitivity, the allergen is first phagocytized by a macrophage and presented to T-cells. The T-cells that cause this type of response had been given the name **delayed-type hypersensitivity T-cells** or T_{DTH} cells, but are now know to be T_H1 cells. T_H1 cells activated by the allergen respond by proliferating, migrate to the area of allergen and then release cytokines, which attract lymphocytes, macrophages and basophils to the tissue. The resulting immune response can cause **extensive tissue damage**.

Delayed type hypersensitivity reactions are familiar to anyone who has suffered after brushing up against *poison ivy* and *poison oak*. In this case, the cause of the reaction is contact with catechols present in the oily leaves of the poison ivy and poison oak plant. Catechols on the skin result in the formation of a characteristic rash with intense itching, swelling and blistering. The catechols are low molecular weight chemicals (haptens) that are capable of combining with a protein to form an antigen. Contact with leaves of the poison ivy plant causes some of the catechols to rub off and stick to lipids in the skin and eventually combine with a skin protein to form the allergen. This then

sensitizes the T_H1 cells. During **initial exposure**, only T_H1 memory cells are created and no dermatitis occurs. In **second and subsequent exposures**, the T_H1 memory cells become active, recruit other immune cells and cause the characteristic rash.

Type IV or Delayed Hypersensitivity can be illustrated by considering the following experiment: First, a guinea pig is injected with a sub-lethal dose of *Mycobacterium tuberculosis* (MT). Following recovery of the animal, injection of a lethal dose of MT under the skin produces only erythema (redness) and induration (hard spot) at the site of injection 1–2 days later. Instead of reinjecting the immunized guinea pig, serum is transfered from this pig to a "naive" (unimmunized) pig. When this second guinea pig is now injected with MT, it dies of the infection.

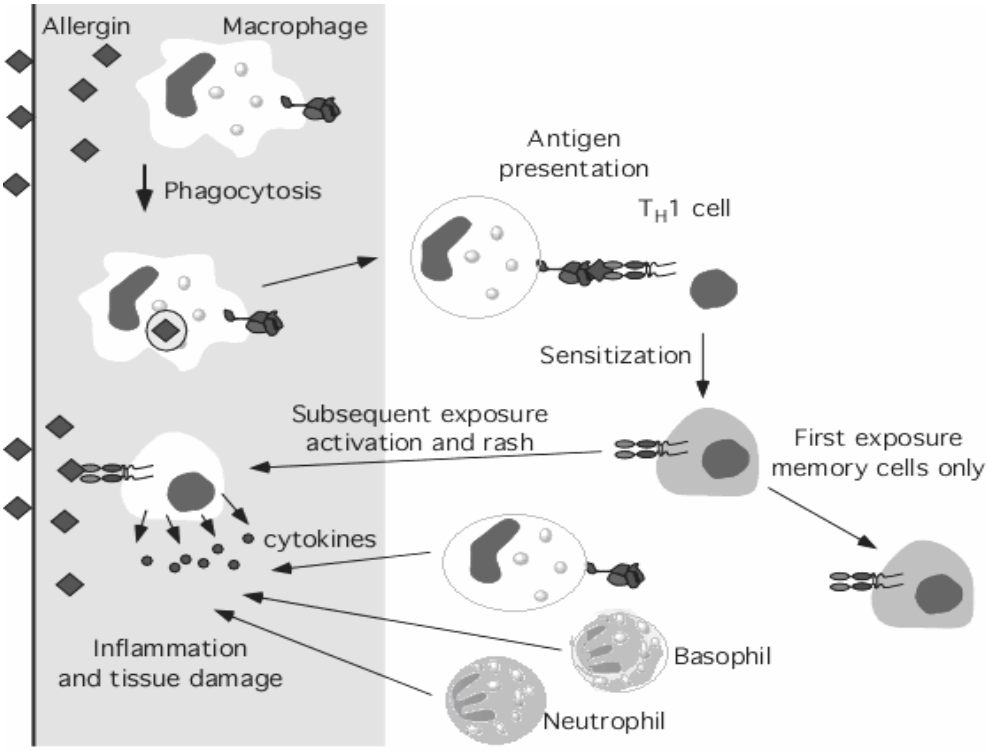


Figure 109 — Cell-mediated hypersensitivity

If immune cells (T-cells and macrophages instead of serum) are transfered from the immunized pig to a second pig, the result is very different; injection of the second pig with MT causes only erythema and induration at the site of injection 1–2 days later. In a separate experiment, if the immunized guinea pig is injected with a lethal dose of *Listeria monocytogenes* (LM) instead of MT, it dies of the infection. However, if the pig is simultaneously injected with both LM and MT, it survives.

These results tell that:

1. The reaction elicited by antigen occurs relatively slowly (hence the name "delayed hypersensitivity").
2. The hypersensitivity is mediated via T-cells and macrophages.
3. The hypersensitivity illustrates both antigen-specific (T-cell) and antigen non-specific (macrophage) characteristics.

Soaps, cosmetics and *metals (especially nickel)* can also cause this type of contact dermatitis in sensitive individuals.

Table 24 — Comparision of different types of hypersensitivity

Charcteristics	Type I (anaphylactic)	Type II (cytotoxic)	Type III (immune complex)	Type IV (delayed type)
Antibody	Ig E	Ig G, M	Ig G, M	None
Antigen	Exogenous	Cell surface	Soluble	Tissues and organs
Response time	15–30 minutes	Minutes-hours	3–8 hours	48–72 hours
Appearance	Weal and flare	Lysis and necrosis	Erythema, edema, necrosis	Erythema and induration
Histology	Basophils and eosinophils	Antibodies and complement	Complement and neutrophils	Monocytes and lymphocytes
Transferred with	Ab	Ab	Ab	T-cells
Examples	Allergic asthma, hay fever	Erythroblastosis, Goodpasture's nephritis	Systemic lupus erythematosus, farmer's lung disease	epidermal (organic chemicals, poison ivy, heavy metals, *etc.*), tuberculin test, granuloma

The archetype of delayed hypersensitivity is the **tuberculin reaction**. When a small dose of tuberculin is injected intradermally in an individual sensitised to tuberculoprotein by prior infection or immunisation, an indurated inflammatory reaction develops at the site within 48–72 hours. In unsensitised individuals, the tuberculin injection provokes no response. The tuberculin test therefore provides useful indication of the state of delayed hypersensitivity (cell mediated immunity) to the bacilli. The tuberculin test differs from the skin test for Type I hypersensitivity not only in the longer interval for appearance but also in its morphology and histology.

Tuberculin type hypersensitivity develops in many infections with bacteria, fungi, viruses and parasites, especially when the infection is subacute or chronic and the pathogen intracellular. A similar hypersensitivity is developed in allograft reaction and in many autoimmune diseases.

Immunoprophylaxis

An important contribution of microbiology to medicine has been immunisation, which is one of the most effective methods of controlling infectious diseases. By systematic active immunisation, many developed countries have virtually eliminated 'vaccine preventable diseases' (VPD) such as diphtheria, pertussis, tetanus, measles, mumps, rubella and poliomyelitis. The global eradication of smallpox, of course, has been the crowning glory of immunisation. **Immunoprophylaxis may be in the form** of 1) routine immunisation, which forms part of basic health care, or 2) immunisation of individuals or selected groups exposed to risk of specific infections.

Routine immunisation

Routine immunisation schedules have been developed for different countries and modified from time to time, based on the prevalence of infectious diseases, their public health importance, availability of suitable vaccines, their cost benefit factors, and logistics. In India, the Expanded Programme on Immunisation (EPI) and the Universal Immunisation Programme (UIP) have been able to afford protection for much of the target population against VPDs. The National Immunisation Schedule in force in India is shown in Table 25.

Table 25 — National Immunisation Schedule (India)

Age	Vaccine
At birth[1]	BCG, OPV-O
6 weeks	BCG[2] DPT-1, OPV-1
10 weeks	DPT-2, OPV-2
14 weeks	DPT-3, OPV-3
9 months	Measles
16-24 months	DPT, OPV
5-6 years	DT[3]
10 years	TT[4]
16 years	TT[4]
For pregnant women[5]	TT-1 or booster
One month after TT-1	TT-2

Note: 1. For institutional births only. OPV-0 is additional, and not to be counted for the primary course of 3 doses starting at 6 weeks. 2. Only for infants not given BCG at birth. 3. A second dose of DT to be given to children with no documentary evidence or history of primary DPT immunisation. 4. A second dose of TT to be given after one month to those with no record or history of prior DPT, DT or TT immunisation. 5. For prevention of tetanus in the neonate primarily, but also in the mother.

In India, EPI and UIP have led to a significant decline in the recorded incidence of VPDs, as well as of infant and child mortality. For example, it has been reported that in 1992 alone, 1.7 million lives of children under five years were saved, as compared to the mortality figures in 1984, the year before UIP was started.

Individual immunisation

Vaccines offered under national programmes are limited by economic considerations and so some important vaccines may be omitted because they are costly. These may be supplemented by individual initiative, whenever possible.

Hepatitis B vaccine: Many developing countries, including India, have high endemicity for this virus. Perinatal transmission and acquisition of the virus infection in the first five years of life are common in such areas, in contrast to low endemic areas where infection is usually acquired in adolescence or adulthood from sexual or household contacts, contaminated needles, blood or blood products or occupational exposure. Besides the morbidity and mortality due to acute and chronic virus infection, chronic carriage which may be very prolonged is itself a serious public health problem. It has also become an economic problem as carriers are denied entry or employment in many foreign countries. Inclusion of the hepatitis B vaccine in routine childhood immunisation will therefore be beneficial. The fact that a quarter to half the adult dose of the vaccine is adequate for children brings down the cost. Till it becomes part of the national immunisation schedule, it would be desirable to have the vaccine administered to as many children and adults as possible by individual immunisation or through voluntary agencies. The recent reduction in cost of the vaccine as a result of indigenous manufacture, has made mass vaccination more feasible.

MMR vaccine: The composite measles-mumps-rubella vaccine is employed in the affluent countries but in the developing countries only the measles vaccine is given at nine months, the earliest age when it is likely to be immunogenic in the presence of maternal antibodies in the baby. Whenever possible, a dose of MMR vaccine may be beneficial at 16–24 months or later, not only to reinforce immunity against measles but also to protect against mumps and **rubella.**

Varicella vaccine: Chickenpox is very mild disease in children, but in adults it can be serious and even fatal. In most parts of the world, chickenpox is very rare in adults, but in some areas in the tropics it is not uncommon. The age of incidence of varicella is reported to be rising. Varicella vaccine had been used for many years in immunocompromised children. Recently, with the development of a more stable and effective vaccine, its scope has been extended for general use for prevention of varicella and herpes zoster. The live attenuated vaccine is recommended as a single subcutaneous dose in children 9 months to 12 years of age, and as 2 doses at an interval of at least 6 weeks, in those older. Pregnancy is a contraindication.

Typhoid vaccine: Typhoid fever continues to be a major public health problem in the developing countries. Immunisation against typhoid is a real need, particularly in view of the spread of drug resistant typhoid strains. The original typhoid vaccine is not widely used because of its uncertain benefit and frequent adverse reactions. Two recent typhoid vaccines, the live oral Gal-E mutant vaccine and the injectable purified Vi polysaccharide vaccine may be acceptable because they offer prolonged protection and are free from reactions. They are recommended for immunisation of those five years old or above and so may be employed at school entry.

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